Universidade de Aveiro

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AVALIAÇÃO ECOTOXICOLÓGICA DE MISTURAS DE PESTICIDAS

ECOTOXICOLOGICAL EVALUATION OF PESTICIDE MIXTURES

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutoramento em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do CESAM e Departamento de Biologia da Universidade de Aveiro) e do Doutor Amadeu Mortágua Velho da Maia Soares (Professor Catedrático do Departamento de Biologia da Universidade de Aveiro).

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o júri

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palavras-chave

Produtos de protecção das plantas, misturas, ecotoxicologia, concentração de adição, acção independente, sinergismo, antagonismo, microcosmos, biomarcadores, plantas, minhocas, isópodes, colêmbolos.

resumo

Neste trabalho o risco da aplicação de moluscicidas (metaldeído e metiocarbe) para o isópode terrestre *Porcellionides pruinosus* foi avaliado usando como parâmetros a mortalidade e biomarcadores de exposição. O tempo até à morte dos isópodes (após contacto com os moluscicidas) foi muito curto, especialmente no caso da exposição ao metiocarbe. Os vários biomarcadores revelaram-se úteis para a compreensão do modo de acção dos dois moluscicidas neste isópode, particularmente o efeito do carbamato metiocarbe na inibição da enzima acetilcolinesterase (AChE). Os efeitos de combinações binárias de três produtos de protecção das plantas (PPP) dimetoato, glifosato e espirodiclofeno foram avaliados testando o comportamento de evitamento de *P. pruinosus*, o sucesso reprodutivo do colêmbolo *Folsomia candida* e o crescimento das plantas *Brassica rapa* e *Triticum aestivum*, usando os dois modelos de referência de concentração de adição (CA) e acção independente (IA). O modelo MIXTOX foi usado para avaliar possíveis desvios (devido a interacções entre os pesticidas) dos dois modelos de referência. Os resultados obtidos permitem constatar que estes PPP quando aplicados segundo a dose recomendada não acarretam efeitos perniciosos para os organismos testados. Foi detectado sinergismo na mistura feita com glifosato e espirodiclofeno no isópode *P. pruinosus* e na mistura com glifosato e dimetoato na planta *T. aestivum*. Um ecossistema terrestre em pequena escala (“STEM”) foi desenvolvido, contendo um solo agrícola mediterrânico. Nestes STEM, minhocas (*Eisenia andrei*), *P. pruinosus*, *B. rapa* e “bait-lamina” foram incorporados no sentido de avaliar os efeitos da aplicação de dimetoato com espirodiclofeno e glifosato com dimetoato. A dose recomendada de aplicação dos PPP, quer na exposição individual quer nas misturas binárias não teve quaisquer efeitos nas espécies testadas. As minhocas foram sensíveis à aplicação conjunta de dimetoato com espirodiclofeno (10 vezes a dose recomendada) na sua distribuição vertical ao longo da coluna do STEM, e foi detectado sinergismo (i.e. mais minhocas escaparam do que a predição feita pelo modelo IA). Em todas as misturas binárias feitas com glifosato e dimetoato registou-se um decréscimo no consumo de “bait-lamina”, indicando sinergismo (menos “bait-lamina” consumidos que o esperado). Dos quatro biomarcadores (Catalase, AChE, GST e LPO) avaliados nos isópodes, verificaram-se diferenças significativas na actividade da enzima AChE (quando dimetoato foi aplicado no solo) e LPO (aumento da actividade devido à aplicação de glifosato e dimetoato).

keywords

Plan protection products, mixtures, concentration addition, independent action, synergism, antagonism, microcosms, biomarkers, plants, earthworms, isopods, collembolans.

abstract

In this work the risk of applying molluscicidal baits (with metaldehyde and methiocarb) to the terrestrial isopod *Porcellionides pruinosus*, was assesses using the time to lethality as well as biomarkers of exposure. The time to death values found in the single exposures to both molluscicides were very low, especially in the case of methiocarb. The use of several biomarkers was a suitable tool to understand the mode of action of these two molluscicides in this isopod species, particularly the effect of the carbamate methiocarb on the inhibition of acetylcholinesterase (AChE) activity. The effects of binary combinations of three plant protection products (PPP), dimethoate, glyphosate and spirodiclofen on the avoidance behaviour on *Porcellionides pruinosus,* the reproductive effort of the collembolan *Folsomia candida* and on the growth pattern of the plants *Brassica rapa* and *Triticum aestivum* were assessed using the two reference models of concentration addition (CA) and independent action (IA). The MIXTOX model was used to detect possible deviations (due to the interaction between pesticides) from the two reference models. The results seemed to corroborate that these PPPs have no detrimental effects when applied at recommended doses. Synergism was detected in the mixture made with glyphosate and spirodiclofen applied to *P. pruinosus* and with the mixture of glyphosate and dimethoate applied to *T. aestivum.* A small-scale terrestrial ecosystem ("STEM") containing a Mediterranean agricultural soil was designed, where earthworms (*Eisenia andrei*), *P. pruinosus, B. rapa* and bait-lamina strips were incorporated to survey the effects of binary combinations of dimethoate with spirodiclofen and glyphosate with dimethoate. The recommended application dose of the PPP did not cause any impairment in the growth pattern of the tested species. Earthworms were sensitive in their depth distribution due to the application dimethoate and spirodiclofen (10 times the recommended dose) tested, and a synergistic pattern, (i.e. worms escaped more than predicted by the IA model) was observed. In all the binary mixtures performed with dimethoate and glyphosate, a decrease in the feeding activity (bait-lamina consumption) of the soil fauna was observed, indicating synergism (less baits eaten than expected).From the four biomarkers assessed in isopods (Catalase, AChE, GST, and LPO), only a significant difference in the AChE (decrease after dimethoate exposure) and LPO activity (increase after exposure to glyphosate and dimethoate) was observed.

We know more about the movement of the celestial bodies than about the soil underfoot.

Leonardo da Vinci

1. GENERAL INTRODUCTION

1.1 Concepts in mixture toxicity

Due to the sometimes contradictory use of concepts in mixture toxicity in scientific literature, in the interest of clarity, we should carefully define key mixture concepts used along this thesis. A mixture can be defined as a "combination of two or more component chemical/compounds to which living organisms may be exposed" either simultaneously or sequentially (McCarty and Bogert, 2006). According to the same authors an "interaction" between two or more compounds occurs when the "level of response produced by any combination of different agents differs from the response expected on the basis of a theoretical model of non-interaction".

The mode of action of a chemical can be defined as "set of biochemical, physiological and behavioural signs that characterize an adverse biological response" in an organism exposed to a stress factor (McCarty, 2002). The term "additivity" should be used when several compounds in a mixture act "without any interaction among them and the total effect does not differ from what can be expected from the effects of the individual agents" (Goldoni and Johansson, 2007). As a result, if additivity fails to explain the joint toxicity this could be the consequence of chemical-chemical interactions, toxicokinetic interactions (e.g. uptake, biotransformation) or toxicodynamic interactions (e.g. at the receptor site inside the organism). These interactions can have as a consequence an effect less than additive (antagonism) or an effect greater than additive (synergism) of the components in the mixture (Lydy et al., 2004).

Plant protection products (PPP) are defined in the plant protection products directive (article 2 of 91/414/EC) as "active substances and preparations containing one or more active substances, put up in the form in which their are supplied to the user, intended to protect plants or plant products against all harmful organisms, or prevent the action of such organisms [...]." All the active substances used and regulated as PPPs are now

considered as being already registered under the European legislation concerning the Registration, Evaluation and Assessment of Chemicals (REACH).

1.2 Models used in mixture toxicity assessment

Two reference models have been used for mixture toxicity assessment: concentration addition (CA) and independent action (IA). Both models have as common ground the concept of non-interaction, since both assume that the components present in the mixture do not interact with each other, meaning that each component does not influence the biological action of the other component present in the mixture (Hewlett and Plackett, 1959).

The concentration addition model is used to evaluate mixtures that are constituted by components that have a common mode of action and will act at the same target site inside the organism (Köneman and Pieters, 1996). According to the definition proposed by Loewe and Muischneck (1926) the CA model stipulates that the components of the mixture act independently but similarly, so that one component can be substituted at a constant ratio of an equi-effective proportion of a second component without altering the toxicity of the mixture. According to this reference model all the components present in the mixture contribute to the overall effect, even if its concentration in the mixture is below the effect threshold (Silva et al., 2002).

Mathematically the CA model can be expressed as:

$$\sum_{i=1}^{n} \frac{ci}{ECxi} = 1$$

where c_i is the concentration of the *i*-th component in the mixture and EC_{x_i} is the effect concentration of component *i* in the mixture (Berenbaum, 1985). The quotient c_i/ECx_i is also referred as the toxic unit (TU) value, which quantifies the contribution of the toxicity of chemical *i* in the mixture of *n* chemicals (Jonker et al., 2005). The contribution of each component can be addressed in terms of the dimensionless TU concept, which is a method of toxicity scaling of the components present in the mixture. The effect

concentration (EC*x*) can be chosen, albeit the level where 50 % of effect is observed (EC_{50}) is normally the most relevant, since it is in the middle of the dose response curve and thus less prone to variability.

The CA reference model has predicted, successfully, the effect of mixtures of similar acting compounds in several toxicology and ecotoxicology studies (Hermens et al., 1984; Deneer et al., 1988; Backhaus et al., 1999; Altenburger et al., 2000; Faust et al., 2001; Junghans et al., 2003; Cleuvers, 2004; Richter and Escher, 2005; Baldwin and Roling, 2008; Porsbring et al., 2010). Concentration addition has been proposed as a possible default model for mixture toxicity assessment schemes in the European Union, due to its predictability power even in mixtures with dissimilar compounds (Boedeker et al., 1993; Lock and Janssen, 2002; Backhaus et al. 2004), and the fact that is more "conservative" than independent action, hence the effects predicted by CA are usually higher (with consequently smaller effect levels) than the effects predicted by IA (Kortenkamp et al., 2009).

The independent action model is used to evaluate mixtures with components that have different modes of action and act upon different target sites in the organisms and it is based on response additivity (Köneman and Pieters, 1996). Bliss (1939) established that according to the IA model the "toxicity of any combination can be predicted from that of the isolated components and from the association of susceptibilities to the two components". Thus, this reference model stipulates that the "effects of two factors [stressors] can be predictable from the response probabilities of the separate factors" taking in consideration the probabilistic independence of the response to each stressor present in the mixture (Piegorsch et al., 1986). Contrary to the CA model, under the independent action concept only the components that cause an effect are considered, thus components below the effects threshold (e.g. EC_0) will not contribute to the toxicity of the mixture (Faust et al., 2003).

Mathematically, the IA model can be expressed as:

$$Y = u \max \prod_{i=1}^{n} qi(ci)$$

where Y is the biological response and qi(ci) is the probability of nonresponse (Jonker et al., 2005). For quantal responses (e.g. avoidance behaviour where the animal either is present or absent from the contaminated soil) this means that the unaffected proportion can be expressed by the probabilities of nonresponse to the toxicants, but for continuous data sets (e.g. shoot length of plants) the probabilities of nonresponse (unaffected proportion) must be multiplied by the maximum value, assumed to be the control value, in order to obtain the mixture toxicity suggested by the IA model (Martin et al., 2009).

The independent action model has been able to predict the effects of dissimilarly acting chemicals in several ecotoxicological and toxicological studies (Van Gestel and Hensbergen, 1997; Backhaus et al., 2000; Payne et al., 2000; Walter et al., 2002; Faust et al., 2004; Jonker et al., 2004). Independent action has also been reported to predict the effects of similar acting compounds (Cedergreen et al., 2008; Syberg et al., 2008). This could be the result of the difficulty in defining the mode of action of chemicals, even for the case of pesticides where the mode of action is usually well established for the target organisms, secondary or subsidiary effects could derive from the designed target effect of the pesticide (Pope, 1999). Nevertheless, the independent action model has been regarded as a possible default model to assess, for example, cancer risk in human toxicology (Kortenkamp et al., 2007).

1.3 Ecotoxicological assessment of pesticide mixtures

It is recognized that the impact of a singe chemical exposure is an improbable event, thus the organisms are exposed to a cocktail of different contaminants in water, air and soil compartments. For example, edaphic organisms in agricultural fields are exposed to several pesticides (herbicides, insecticides, fungicides), which can be applied at the same time or in consecutive days (Santos et al., 2010b). Therefore, it seems pertinent to focus at a starting point on the risk that a single compound can pose to a given organism and address the effects that the application of several compounds can pose to non-target organisms.

Assessing the effects of pesticide mixtures, due to the almost infinite possibility of combinations of pesticides already present in soil ecosystems and the forthcoming new chemical entering the market, would be at best an unfortunate objective to anyone decided to undertake such a task. The European legislation (REACH) foresees mixture toxicity assessment only in specific cases of mixtures and at specific stages of non-target organism life cycle, although the European Union interest in the development and production of risk assessment schemes contemplates multi-pollutant exposure scenarios (Syberg et al., 2010). It also should be pointed out that the available legislation concerning the introduction of new chemicals contemplates the evaluation of toxic mixtures only for the aquatic compartment (EC, 2008).

Although most studies dealing with pesticide mixtures have been published in the research area of aquatic toxicology, the publication of research papers about the joint effects of pesticides in terrestrial systems has flourished in the past years. The assessment of the effects of pesticides mixtures has been reported for a great variety of organisms such as mites (Chapman and Penman, 1980), insects (Ascher et al., 1986; Van der Greet et al., 2000), carabid beetles and cockroaches (Fischer, 1992), lepidopters (Ahmad, 2004), rats (Gordon et al., 2006), plants (An and Lee, 2007; Chao et al., 2007), enchytraeids (Loureiro et al., 2009), nematodes (Jonker et al., 2004; Martin et al., 2009), earthworms (Lydy and Linck, 2003; Gomez-Eyles et al., 2009), birds (Chen et al., 2009), snails (Druart et al., 2010), isopods (Loureiro et al., 2009; Santos et al., 2010b), and collembola (Martikainen et al., 1998; Broerse and Van Gestel, 2010). In the majority of the studies cited above, in some of the concentrations/combinations tested or in all the concentration range, an interaction between the pesticides was observed, reflecting antagonism or synergism of the mixture toxicity.

For binary mixtures, the MIXTOX model proposed by Jonker and coworkers (2005) permit to detect deviations (interactions) from the two reference models of concentration addition and independent action. This descriptive model not only allows evaluating if synergism and antagonism occurs in the binary mixture, but also the description of two more complex deviations, namely dose ratio (deviation is dependent of the ratio of the two components of the mixture) and dose level dependent deviation (deviation is dependent of the dose of each component in the mixture).

1.4 The use of microcosms in terrestrial ecotoxicology

In recent years, enclosed model ecosystems (microcosms) have been developed in order to achieve a greater realism in the evaluation of pesticides and other xenobiotics (Van den Brink et al., 2005). Terrestrial microcosms are by definition, "soil units that contain multiple organisms from different trophic levels" (Burrows and Edwards, 2002). There are considered a useful tool for evaluate the effects of harmful compounds to soil organism "in their natural environment, and for tracking the effects of [the] responses at higher levels of biological organization, such as regulation of community structure, decomposition processes and primary production" (Lukkari et al., 2006). Since most ecotoxicological studies rely on the assessment of one single-test species (e.g. ISO guidelines), microcosms have been proposed as a functional tool to assess the effects of combined pollutants to several non-target species (Alonso *et al.*, 2009). Although some prudence should be taken in consideration when extrapolating the results obtained in microcosms to field conditions (Kampichler et al., 2001), the fact is that microcosms can represent a step forward on the presentation of more "realistic" results in soil ecotoxicology. In the present work a small-scale terrestrial ecosystem (STEM) was developed, as a surrogate of real scenarios of exposure in agricultural fields.

1.5 Tests organism and test chemicals

In the present study three invertebrate species (the collembolan *Folsomia candida* Willem, the earthworm *Eisenia andrei* Bouché, and the isopod *Porcellionides pruinosus* Brandt) and two plant species (the monocotyledonous *Triticum aestivum* L. and the dicotyledonous *Brassica rapa* L.*)* were used in the several tests performed.

Collembolans are part of soil ecosystems and their abundance and diversity has been used to assess the effects of different pollutants on soils (Moe et al., 2001), hence the

standardized protocols available for the assessment of its reproduction success (ISO, 1999) and avoidance behaviour (ISO, 2008b). The collembolan species *Folsomia candida* is one of the most used in ecotoxicology studies due to the ease in keeping in laboratory controlled conditions, relative short generation time and simplicity in manipulating along the experimental procedure (Ronday and Houx, 1996). Due to their contribution to the decomposition processes and organic matter break down, it seems important to study the effects that agricultural practices (in terms of pesticide application) may bring to their population levels (Eaton, 2006).

Based on their biomass, earthworms are the predominant group of soil invertebrates, and are known to play an important role in improving soil quality and promoting plant productivity (Morgan et al, 2002). In addition, earthworms have an important role as primary decomposers of organic matter, mixing of soil components, and are an important food source for other animals. Fields with earthworm populations can absorb water at a rate 4 to 10 times higher than fields lacking worm tunnels (Edwards and Bohlen, 1996). Moreover, they provide soil aeration and soil drainage which in turn increases the source of nutrients and enzymes for soil microorganisms (Kula & Larink 1998). The earthworm *Eisenia andrei* has been considered a good test-species for the toxicity assessment of different types of chemicals (Van Gestel et al., 1992), as is attested by the standardized protocols available for the assessment of its reproduction success (ISO, 1998) and avoidance behaviour (ISO, 2008a) to the application of chemicals.

Terrestrial isopods are in certain ecosystems, namely in temperate habitats, the dominant components of the macrodecomposer community structure; they feed on decayed organic matter (litter) and are considered key regulators of such soil ecosystem functions as litter decomposition and nutrient cycling (Paoletti and Hassall, 1999). Several studies have been published with *Porcellionides pruinosus*, and this species has proven to be a good indicator of pesticide and metal contamination in soils (Loureiro et al., 2005; Loureiro et al., 2009; Santos et al., 2010b).

Terrestrial plants have a paramount importance in ecosystems, due to their role in nutrient cycling, stabilization of soil (avoiding erosion), and primary production as well as providers of food and habitat to other organisms (Boutin et al., 1995). Therefore, the potential pernicious effects that pesticide application may pose to non-target plants can

change the equilibrium in agricultural ecosystems (Lydon and Duke, 1989). The two plant species used in this work have been employed in the ecotoxicological assessment of several xenobiotics (e.g. Günther and Pestemer, 1990; Kristen, 1997), and as a result guidelines concerning the evaluation of chemicals contain these two species as surrogates for terrestrial higher plants (ISO, 1995; ISO, 2004).

Five different plant protection products were used in the tests performed: the molluscicidal baits metaldehyde and methiocarb, the insecticide dimethoate, the herbicide glyphosate and the acaricide spirodiclofen. In all experiments the commercial formulations were used, in compliance with regulatory application recommendations.

The molluscicides methiocarb and metaldehyde are among the most used in the chemical control of snail and slugs, comprising almost 90% of the sales of molluscicides in the European territory (Henderson and Triebskorn, 2002). Metaldehyde induces severe alterations and ultrastructural destruction in mucocytes which leads to dehydration and subsequent death of molluscs (Triebskorn et al. 1998), whereas methiocarb acts upon the central nervous system inhibiting acetylcholinesterase (AChE), which can cause overstimulation of the nervous system and ultimately the death of the animal (Bailey, 2002). The organophosphorous insecticide dimethoate is one of the most used PPP in Europe in agricultural fields (Eurostat, 2007), and its mode of action to invertebrates is through the inhibition of AChE (Matikainen, 1996). In plants it has been shown to be through the inhibition of the synthesis or action of hydrolytic enzymes inside the seeds (Chopra and Nandra, 1969). The post-emergence herbicide glyphosate, which is one of the most applied PPP in European countries (Eurostat, 2007), inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in plants (Steinrucken, and Amrhein, 1980). Spirodiclofen is a selective, non-systemic acaricide from the novel class of tetronic acid derivatives, which interferes with lipid biosynthesis by inhibiting acetyl-Coa carboxylase in acari, and no effects in terrestrial plants were observed in previous laboratory testing to its commercial release (Wolf and Schnorbach, 2002).

1.6 Conceptual framework

This thesis is divided in seven chapters, including the current General Introduction (Chapter 1), five chapters (Chapters 2 to 6) describing the main results of the toxic assessment of the application of pesticides, and a General Discussion of the main results obtained in the work performed (Chapter 7).

In this study the effects of PPPs in both single and binary mixtures were assessed in different endpoints as survival, biomarkers and avoidance behaviour (*Porcellionides pruinosus*), reproduction success (*Folsomia candida*), vegetative growth and germination success (*Triticum aestivum*, *Brassica rapa*), and depth distribution and weight variation (*Eisenia andrei*). This work covered the effects of pesticide application from the assessment of differences in biomarkers activity in isopods through the effects of pesticide application in a multispecies microcosm's apparatus, mimicking real scenarios of exposure. Therefore the studies performed span from cellular responses (enzymatic biochemical indicators) to the ecological interactions between several edaphic organisms exposed at the same time (microcosm design of small-scale terrestrial ecosystems).

In chapter two: "Toxic effects of molluscicidal baits to the terrestrial isopod *Porcellionides pruinosus* (Brandt, 1833)", the effects of two molluscicides, metaldehyde and methiocarb, were assessed at lethal levels along time, on the activity of three enzymatic biomarkers (AChE, Glutathione-*S*-transferase, and Catalase) on single and joint action to this terrestrial isopod.

In chapter three: "Joint effects of three plant protection products to the isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*", the assessment of the avoidance behaviour of *P. pruinosus* after 48 hours of exposure and the reproduction success of *F. candida* after 28 days of exposure were assessed in both single and binary combinations of the three PPPs using a LUFA 2.2 soil as exposure medium. For the assessment of pesticides' combination, the MIXTOX tool was used to evaluate if the reference models CA and IA described the data obtained or if deviations (interactions) between the PPPs occurred in the binary mixtures.

In chapter four: “The joint toxicity of three plant protection products to *Triticum aestivum* (L.) and *Brassica rapa* (L.)”, the application of dimethoate, glyphosate and spirodiclofen was assessed in terms of plant biomass production (fresh weight) and vegetative growth (shoot length) of the two plant species after approximately 21 days of exposure in both single applications and binary mixtures of the three PPPs using a LUFA 2.2 soil. Again, for the evaluation of the joint effects, the MIXTOX model was used in order to see if the reference models CA and IA described the data or if deviations (interactions) between the PPPs occurred in the binary mixtures.

In chapter five: “Evaluation of the joint effect of dimethoate and spirodiclofen to plants and earthworms in a designed microcosm experiment”, a designed small-scale terrestrial ecosystem was used with a Mediterranean agricultural soil where PPPs were applied. The objectives of this work were to evaluate if a microcosm experiment could mimic what happens in real exposure scenarios of application of PPPs and to compare, when possible, with results from single test species. For that it was assessed the combined effects of the two pesticides to the earthworm *Eisenia andrei* (in terms of depth distribution along the soil column, and weigh change) and the turnip species *Brassica rapa* (in terms short length and fresh weight) using the concept of independent action. The observations were made after 28 days of exposure to the pesticides in both single exposures (to the respective field dose, 5 and 10 times the field dose of each pesticide) and binary mixtures made with the same range of concentrations.

In chapter six: “Evaluation of the joint effect of glyphosate and dimethoate using a small-scale terrestrial ecosystem”, the same experimental units were used to assess the effects of the two PPP on the depth distribution and weight change of *E. andrei*, on the growth, weight and germination success of *B. rapa*, on the enzymatic biomarkers of *P. pruinosus* and on the feeding activity of soil fauna (bait-lamina test). These endpoints were measured after 7 days of exposure (bait-lamina test) and 28 days of exposure to the pesticides in both single exposures (to the respective field dose, 5 and 10 times the field dose of each pesticide) and binary mixtures made with the same range of concentrations.

In chapter seven: “General discussion”, the main results are discussed in terms of biological significance and practicability of the several experimental procedures performed. In conclusion, an encompassing perspective of the suitability of assessing

pesticide mixtures instead of the single application approach usually followed in ecotoxicology studies was accomplished.

References

Ahmad, M., 2004. Potentiation/antagonism of deltamethrin and cypermethrin with organophosphate insecticides in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctudridae). Pest. Biochem. Phisyol. 80 (1), 31-42.

Alonso, E., Nunez, M.G., Carbonell, G., Fernández, C., Tarazona, J.V., 2009. Bioaccumulation assessment via an adapted multi-species soil system (MS.3) and its application using cadmium. Ecotox. Environ. Safe. 72, 1038-1044.

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H., 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: mixtures composed of similarly acting compounds. Environ. Toxicol. Chem. 19 (9), 2341-2347.

An, Y.-J-., Lee, W.-M., 2007. Decreased toxicity to terrestrial plants associated with a mixture of methyl *tert*-butyl ether and its metabolite *tert*-butyl alcohol. Environ. Toxicol. Chemi. 26 (8), 1711-1716.

Ascher, K.R.S., Eliyahu, M., Ishaarya, I., Zur, M., Ben-moshe, E., 1986. Synergism of pyrethroid-organophosphate mixtures in insects and their toxicity against *Spodoptera littoralis* larvae. Phytoparasitica 14 (2), 101-110.

Backhaus, T., Scholze, M., Grimme, L.H., 1999. The single substance and mixture toxicity of quinolones to the bioluminescent bacterium *Vibrio fischeri*. Aquatic Toxicology 49, 49-61.

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H., 2000. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *Vibrio fischeri*. Environ. Toxicol. Chem. 19(9), 2348-2356.

Backhaus, T., Arrhenius, A., Blanck, H., 2004. Toxicity of a mixture of dissimilarly acting substances to natural algal communities: predictive power and limitations of independent action and concentration addition. Environ. Sci. Technol. 38, 6363-6370.

Baldwin, W.S., Roling, J.A., 2008. A concentration addition model for the activation of the constitutive androstane receptor by xenobiotic mixtures. Toxicological Sciences 107(1), 93-105.

Bailey, S.E.R., 2002. Molluscicidal baits for control of terrestrial gastropods. In: Barker, GM (ed), Molluscs as crop pests, CABI Publishing, Wallingford, U.K., pp 33–54.

Berenbaum, M.C., 1985. The expected effect of a combination of agents: the general solution. J. Theor. Biol. 114, 413 – 431.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Annals of Applied Biology. 26 (3), 585-615.

Boedeker, W., Drescher, K., Altenburger, R., Faust, M., Grimme, L.H., 1993. Combined effects of toxicants: the need and soundness of assessment approaches in ecotoxicology. Sci. Total Environ. 134 (2), 931-935.

Boutin, C., Freemark, K.E., Keddy, C.J., 1995. Overview and rationale for developing regulatory guidelines for nontarget plant testing with chemical pesticides. Environ. Toxicol. Chem. 14 (9), 1465-1475.

Broerse, M., Van Gestel, C.A.M., 2010. Mixture effects of nickel and chlorpyrifos on *Folsomia candida* (Collembola) explained from development of toxicity in time. Chemosphere 79(9), 953-957.

Burrows, L.A., Edwards, C.A., 2002. The use of integrated soil microcosms to predict effects of pesticides on soil ecosystems. Eur. J. Soil Biol. 38, 245-249.

Cedergreen, N., Christensen, A.M., Kamper, A., Kdsk, P., Mathiassen, S.K., Streibig, J.C., Sørensen, H., 2008. A review of independent action compared to concentration addition as reference model for mixtures of compounds with different molecular target sites. Environ. Toxicol. Chem. 27 (7), 1621-1632.

Chao, L., Zhou, Q., Chen, S., Cei, S., Wang, M., 2007. Single and joint stress of acetochlor and Pb on three agricultural crops in northeast China. J. Environ. Sci. 19 (6), 719-729.

Chapman, R.B., Penman, D.R., 1980. The toxicity of mixtures of a pyrethroid with organophosphorous insecticide to *Tetranychus urticae* Koch. Pest. Sci. 11 (6), 600-604.

Chen,F., Zhang, X., Mai, B., Sun, Q., Song, J., Liu, X., Zeng, E.L., Hale, R.C:, 2009. Polychlorinated biphenyls and organochlorine pesticides in various bird species from northern China. Environ. Poll. 157 (7), 2023-2029.

Chopra, S.I., Nandra, K.S., 1969. Effect of thiometon on the germination of sarson (*Brassica campestris* Linn. Var Brown sarson). Journal of Agricultural and Food Chemistry, 17, 805-806.

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotox. Environ. Safe. 59(3), 309-315.

Deneer, J.W., Sinnige, T.L., Seinen, W., Hermens, J.L.M., 1988. The joint acute toxicity to Daphnia magna of industrial organic chemicals at low concentrations. Aquatic Toxicology 12 (1), 33-38.

Druart, C., Scheifer, R., Vanfleury, A., 2010. Towards the development of an embriotoxcicity bioassay with terrestrial snail: Screening approach for Cd and pesticides. J. Hazard. Mat. 184, 26-33.

Eaton, R.J., 2006. Collembola population levels 7 years after installation of the North Carolina Long Term Soil Productivity Study. Pedobiologia 50, 301-306.

EC, 2008. Regulation 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures. Official J. Eur. Union.

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms. Chapman and Hall, London, 426 pp.

Eurostat,2007. The use of plant protection products in the European Union. Data 1992-2003. Eurostat Statistical books, 2007 edition.

Faust, M., Altenburger, R., Backhaus, T., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vigni, M., Grimme, L.H., 2001. Predicting the joint algal toxicity of multi-component *s*-triazine mixtures at low-effect concentrations of individual toxicants. Aquatic Toxicology 56, 13-32.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vigni, M., Grimme, L.H., 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquatic Toxicology 63 (1), 43-63.

Fischer, S.W., 1992. Joint action between binary mixtures of chlordimeform and insecticides. Ecotox. Environ. Safe. 23 (1), 11-21.

Goldoni, M., Johansson, C., 2007. A mathematical approach to study combined effects of toxicants in vitro: Evaluation of the Bliss independence criterion and the Loewe additivity model. Toxicology in Vitro 21, 759-769.

Gomez-Eyles, J.L., Svendsen, C., Lister, L., Martin, H., Hodson, M.E., Spurgeon, D.J., 2009. Measuring and modelling mixture toxicity of imidacloprid and thiacloprid on *Caenorhabditis elegans* and *Eisenia fetida*. Ecotox. Env. Saf. 72 (1), 71-79.

Gordon, C.J., Herr, D.W., Genning, C., Graff, J.E., McMurray, M., Stork, L., Coffey, T., Hamm, A., Mack, C.N., 2006. Thermoregulatory response to an organophosphate and carbamate insecticide mixture: testing the assumption of dose additivity. Toxicology 217(1), 1-13.

Günther, P., Pestemer, W., 1990. Risk assessment of selected xenobiotics by bioassay methods with higher plants. Environ. Manage. 14 (3), 381-388.

Henderson I, Triebskorn R (2002) Chemical control of terrestrial gastropods. In: Barker GM (ed) Molluscs as crop pests, CABI Publishing, Wallingford, U.K., pp 1–31.

Hermens, J., Leeuwangh, P., Musch, A., 1984. Quantitative structure-activity relationships and mixture toxicity studies of chloro- and alkylanilines at an acute lethal toxicity level to the guppy (*Poecillia reticulata*). Ecotox. Environ. Safe. 8(4), 388-394.

Hewlett, P.S., Plackett, R.L., 1959. A unified theory for quantal responses to mixtures of drugs: non interactive action. Biometrics 15, 591-609.

ISO (International Organization for Standardization), 1995. Soil quality – Determination of the effects of pollutants on soil flora – Part 2: Effects of chemicals on the emergence and growth of higher plants. ISO/DIS 11269-2, Geneve, Switzerland.

ISO (International Organization for Standardization), 1998. Soil quality – Effects of pollutants on earthworms (*Eisenia fetida*) – Part 2: Determination of effects on reproduction. ISO/DIS 11268-2, Geneve, Switzerland.

ISO (International Organization for Standardization), 1999. Soil quality – inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants. ISO/DIS 11267, Geneva, Switzerland.

ISO (International Organization for Standardization), 2004. Soil quality – Biological methods – Chronic toxicity in higher plants. ISO/DIS 22030, Geneve, Switzerland.

ISO (International Organization for Standardization), 2008. Soil quality – avoidance test for determining the quality of soils and effects of chemicals on behaviour – Part 1: Test with earthworms (*Eisenia feti*da and *Eisenia andrei*). ISO/DIS 17512-1, Geneva, Switzerland.

ISO (International Organization for Standardization), 2008. Soil quality – avoidance test for determining the quality of soils and effects of chemicals on behaviour – Part 2: Test with collembolans (*Folsomia candida*). ISO/DIS 17512-2, Geneva, Switzerland.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.M., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ. Toxicol. Chem. 24, 2701-2713.

Jonker, M.J., Sweijen, R., Kammenga, J.M., 2004. Toxicity of simple mixtures to the nematode *Caenorhabidtis elegans* in relation to soil sorption. Environ. Toxicol. Chem. 23, 480-488.

Junghans, M., Backhaus, T., Faust, M., Scholze, M., Grimme, L.H., 2003. Predictability of combined effects of eight chloroacetanilide herbicides on algal reproduction. Pest Manag. Sci. 59 (10), 1101-1110.

Kampichler, C., Brukner, A., Kandeler, E., 2001. Use of enclosed model ecosystems in soil ecology: a bias towards laboratory research. Soil Biol. Biochem. 33, 269-275.

Köneman, W.H., Pieters, N.M., 1996. Confusion of concepts in mixture toxicology. Food Chem. Toxicol. 34, 1025-1031.

Kortenkamp, A., Faust, M., Scholze, M., Backhaus, T., 2007. Low-level exposure to multiple chemicals: reasons for human health concerns? Environ. Health Perspect. 115, 106-114.

Kortenkamp, A., Backhaus, T., Faust, M., 2009. State of the art of mixture toxicity. Final report. European Commission. Study contract number 070307/2007/485103/ETU/D.1.

Kristen, U., 1997. Use of higher plants as screens for toxicity assessment. Toxicol. Vitro 11, 181-191.

Kula, H., Larink, O., 1998. Tests on the earthworms *Eisenia foetida* and *Aporrectodea caliginosa*. In Handbook of soil invertebrate toxicity tests. (Eds. Lokke H., Van Gestel C.A.M.), Wiley, New York, pp. 95-112.

Lock, K., Janssen, C.R., 2002. Mixture toxicity of zinc, cadmium, copper, and lead to the potworm *Enchytraeus albidus*. Ecotox. Environ. Safe. 52(1), 1-7.

Loewe, S., Muischnek, H., 1926. Effect of combinations: mathematical basis of problem. N-S. Arch. Ex. Path. Ph. 114, 313–326.

Loureiro, S., Soares, A.M.V.M., Nogueira, A.J.A., 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ. Pollut. 138, 121-131.

Loureiro, S., Amorim, M.J.B., Campos, B., Rodrigues, S.M.G., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157 (2), 625-632.

Lydon, J., Duke, S.O., 1989. Pesticide effects on secondary metabolism of higher plants. Pest. Sci. 25 (4), 361-373.

Lydy, M.J., Linck, S.L., 2003. Assessing the impact of triazine herbicides on organophosphate insecticide toxicity to the earthworm *Eisenia fetida*. Arch. Environ. Contam. Toxicol. 45, 343-349.

Lydy, M., Belden, J., Wheelock, C., Hammock, B., Denton, D., 2004. Challenges in regulating pesticide mixtures. Ecology and Society 9(6), 1.

Lukkari, T., Teno, S., Väisänen, A., Haimi, J., 2006. Effects of earthworms on decomposition and metal availability in contaminated soil: microcosm studies of populations with different exposure histories. Soil Biology and Biochemistry 38, 359-370

Martikainen, E., 1996. Toxicity of dimethoate to some soil animal species in different soil types. Ecotoxicol. Environ. Saf. 33 (2), 128–136.

Martikainen, E., Haimi, J., Ahtiainen, J., 1998. Effects of dimethoate and benomyl on soil organisms and soil processes – a microcosm study. App. Soil Ecol. 9, 381-387.

Martin, H.L., Svendsen, C., Lister, L.J., Gomez-Eyles, J.L., Spurgeon, D.J., 2009. Measurement and modelling of the toxicity of binary mixtures in the nematode *Caenorhabidtis elegans* – a test of independence action. Environ. Toxiol. Chem. 28 (1) , 97-104.

McCarty, L.S., 2002. Issues at the interface between ecology and ecotoxicology. Toxicology 181-182, 497-503.

McCarty, L.S., Bogert, C.J., 2006. Review of the toxicity of chemical mixtures containing at least one organochlorine. Regulatory Toxicology and Pharmacology 45, 104-118.

Moe, S.J., Stenseth, N.C., Smith, R.H., 2001. Effects of a toxicant on population growth rates: sublethal and delayed responses in blowfly populations. Funct. Ecol. 15, 712–721.

Morgan A.J., Evans M., Winters C., Gane M., Davies M.S., 2002. Assaying the effect of chemical ameliorants with earthworms and plants exposed to a heavily polluted metalliferous soil. Eur. J. Soil Biol. 38, 323-327.

Paoletti, M.G., Hassall, M., 1999. Woodlice (Isopoda:Oniscidea): their potential for assessing sustainability and use as bioindicators. Agriculture, Ecosystem and Environment 74, 157-165.

Payne, J., Rajapakse, N., Wilkins, M., Kortenkamp, A., 2000. Prediction and assessment of the effects of mixtures of four xenoestrogens. Environ. Health Persp. 108 (10), 983-987.

Piegorsch, W.W., Weinberg, C.R., Haseman, J.K., 1986. Testing for simple independent action between two factors for dichotomous response data. Biometrics 42, 413-419.

Pope, C.N., 1999. Organophosphorous pesticides: do they all have the same mechanism of toxicity? J. Toxicol. Environ. Health B 2, 161-181.

Porsbring, T., Backhaus, T., Johansson, P., Kuylenstierna, M., Blanck, H., 2010. Mixture toxicity from photosystem II inhibitors on microalgal community succession is predictable by concentration addition. Environ. Toxicol. Chem. 29 (12), 2806- 2813.

Richter, M., Escher, B.I., 2005. Mixture toxicity of reactive chemicals by using two bacterial growth arrays as indicators of protein and DNA damage. Environ. Sci. Technol. 39(22), 8753-8761.

Ronday, R., Houx, N.W.H., 1996. Suitability of seven species of soi-inhabiting invertebrates for testing toxicity of pesticides in soil pore water. Pedobiologia 40, 106-112.

Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing" – eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ. Sci. Technol. 36, 1751-1756.

Santos, M.J.G., Ferreira, N.G.C., Soares, A.M.V.M., Loureiro, S., 2010a. Toxic effects of molluscicidal baits to the terrestrial isopod *Porcellionides pruinosus* (Brandt, 1833). J. Soils Sed. 10 (7), 1335-1343.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2010b. Joint effects of three plant protection products to the isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. Chemosphere 80 (9), 1021-1030.

Steinrucken, H. C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolylpyruvyl-shikimic acid-3-phosphate. Biochem. Biophys. Res. Comm. 94:1207–1212.

Syberg, K., Elleby, A., Pedersen, H., Cedergreen, N., Forbes, V.E., 2008. Mixture toxicity of three toxicants with similar and dissimilar modes of action to *Daphnia magna*. Ecotox. Environ. Safe. 69, 428 – 436.

Syberg, K., Jensen, T.S., Cedergreen, N., Rank, J., 2010. On the use of mixture toxicity assessment in REACH and the water framework directive: a review. Human Ecol. Risk Assess: Int. J. 15 (6), 1257-1272.

Triebskorn, R., Christensen, K., Heim I., 1998. Effects of orally and dermally applied metaldehyde on mucus cells of slugs (*Deroceras reticulatum*) depending on temperature and duration of exposure. J Molluscan Stud 64:467–487.

Van den Brink, P., Tarazona, J.V., Solomon, K.R., Knacker, T., Van den Brink, N.M., Brock, T.C., Hoogland, J.P., 2005. The use of terrestrial and aquatic microcosms and mesocosms for the ecological risk assessment of veterinary medicinial products. Environ. Toxicol. Chem. 24, 820-829.

Van der Greet, H.G., Greve, G.D., Boivin, M-H., Kraak, M.H.S., Van Gestel, C.A.M., 2000. Mixture toxicity of copper and diazinon to larvae of the mayfly *(Ephoron virgo)* judging additivity at different effect levels. Environ. Toxicol. Chem. 19 (12), 2900-2905.

Van Gestel, C.A.M., Dirven-Van Breemen, E.M., Baerselman, R., Emans, H.J.B., Janssen, J.A.M., Posthuma, R., Van Vliet, P.J.M., 1992. Comparison of sublethal and lethal criteria for nine different chemicals on standardized toxicity test using the earthworm *Eisenia andrei*. Ecotox. Environ. Safe 23 (2), 206-220.

Van Gestel, C.A.M., Hensbergen, P.J., 1997. Intercation of Cd and Zn toxicity for Folsomia candida Willem (Collembola:Isotomidae) in relation to bioavailability in soil. Environ. Toxicol. Chem. 16, 1177-1186.

Walter, H., Consolaro, F., Gramatica, P., Scholze, M., Altenburger, R., 2002. Mixture toxicity of priority pollutants at no observed effects concentrations (NOECs). Ecotoxicology 11, 299-310.

Wolf, C., Schnorbach, H.-J., 2002. Ecobiological profile of the acaricide spirodiclofen. Bayer Crop Sci J. 55 (3), 177-906.

2. TOXIC EFFECTS OF MOLLUSCICIDAL BAITS TO THE TERRESTRIAL ISOPOD *PORCELLIONIDES PRUINOSUS* (BRANDT, 1833)

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Abstract

Methiocarb and metaldehyde are the most common molluscicides applied in agricultural and horticultural fields in Portugal and elsewhere in Europe. The application of molluscicidal baits to control slug and snail populations can pose a threat to non-target organisms like terrestrial isopods, because they are detritivorous and may feed on the toxic baits applied to the soil surface. The aim of this work was to evaluate the effects and understand the modes of action of these molluscicides to terrestrial isopods. In this study, the terrestrial isopod *Porcellionides pruinosus* was exposed to these two molluscicides and the time to lethality evaluated. Biochemical indicators (biomarkers) are known to provide early warning signs of environmental pollution or stress conditions to the organisms, by measuring cellular or molecular responses of the target organism to xenobiotic agents. Therefore to evaluate modes of action and effects and also to see if biomarkers can be used as early warning tools in molluscicidal exposures, three different enzymes, glutathione *S*-transferase (GST), acetylcholinesterase (AChE) and catalase (CAT) were analyzed upon single exposures and binary mixtures tests. These two molluscicides showed to be of extreme concern regarding terrestrial isopods as all animals died after 24h of exposure to methiocarb and only 20% survived after 56h of exposure to metaldehyde. Results indicate that the carbamate methiocarb inhibited significantly AChE activity, but no effects were observed in CAT and GST levels. The exposure to metaldehyde had no effects on AChE, but a decrease in GST activity as well as a general increase in CAT activity was observed at the higher exposure period tested (32h). The combined exposure of the two molluscicides resulted in a general decrease in AChE and CAT activity, but no visible effects were observed in terms of GST activity. The LT50 values found in the single exposures to both molluscicides were very low, especially in the case of the carbamate methiocarb. The use of several biomarkers was a suitable tool to understand the mode of action of these two molluscicides in this isopod species.

2.1. Introduction

The chemical control of snails and slugs is mainly carried out with the use of molluscicidal pellets. The most commonly used molluscicides are metaldehyde and the carbamate methiocarb, which represent more than 90% of all European sales for molluscicides (Henderson and Triebskorn 2002). However, the use of these pellets can represent a hazard to non-target soil invertebrates such as woodlice that can feed on the pellets and thus be poisoned by these pesticides (Bailey 2002; Bieri et al. 2003). In agricultural fields molluscicides baits are randomly distributed in the soil surface (by manual or mechanical dispersion) in a way that in some surface areas the number of baits available will be higher than the established recommended dose. The application of molluscicides in some cultures (vineyards and orchards) implies several applications within 14 days safety interval. Moreover in some vegetable crops (e.g. horticultural) baits are applied near the stem in agglomerates, to prevent snails and slugs from attacking the aerial part of the plants. Looking at this, it seems plausible to admit that isopods could encounter more than the recommended dose of baits (granules per area).

The modes of action of these two molluscicides in snail and slugs are well documented, with metaldehyde inducing severe alterations and ultrastructural destruction in mucocytes which leads to dehydration and subsequent death (Triebskorn et al. 1998), whereas methiocarb acts upon the central nervous system inhibiting acetylcholinesterase (AChE), which can cause overstimulation of the nervous system and ultimately the death of the animal (Engenheiro et al. 2005). Terrestrial isopods live on the surface layer of the soil, and are detritivorous species responsible for nutrient recycling in soil ecosystems (Zimmer 2002). *Porcellionides pruinosus* has been used in ecotoxicological tests (Loureiro et al. 2005; Loureiro et al., 2002) and is considered a good test-species to evaluate pernicious effects of xenobiotics (Loureiro et al. 2009).

Biochemical indicators, known as biomarkers, can serve as early warning signs of environmental pollution or stress indication to soil organisms, and can be divided in three classes: exposure biomarkers, effect biomarkers, and susceptibility biomarkers (Schlenk 1999). Biomarkers of exposure are related with cellular or molecular responses indicating

an interaction between an organism and a xenobiotic agent (Roberts and Oris 2004). Several studies have been made using biomarkers as tools to assess the effects of different pollutants to terrestrial isopods, as heavy metals (Köhler et al. 1996), PAHs (Stroomberg et al. 1999), organochlorine pesticides (Köhler et al. 1999), organophosphorous pesticides (Stanek et al. 2006) or titanium nanoparticles (Jemec et al. 2008).

Three enzymes were chosen to perform this study, based on their specific action, relevance and sensitivity to xenobiotic compounds. Acetylcholinesterase is responsible for breaking down acetylcholine, an enzyme that is known to be inhibited by organophosphorous pesticides (OP) and carbamates. These pesticides can bind to the hydroxyl group of the functional part of this enzyme, resulting in a phosphorylated enzyme that has no activity, and hence cannot hydrolyze the substrate acetylcholine (Fulton and Key 2001). Glutathione S-transferase (GST) is a phase II detoxification enzyme, which is involved in the cellular detoxification of several chemicals, catalysing the conjugation of reduced glutathione to several xenobiotic agents (Hayes and Pulford, 1995), and is useful as an indicator of the effects of fungicides and insecticides (Schreck et al. 2008). Catalase (CAT) catalyzes the decomposition of hydrogen peroxide derived from the formation of other Reactive Oxygen Species (ROS), such as superoxide or hydroxyl radicals that were derived from Phase I detoxification processes and led by the cytochrome P450 enzyme (Brown et al. 2004).

The aim of this study was to evaluate the effects of two molluscicides on the terrestrial isopod *Porcellionides pruinosus* (Brandt 1883). This assessment was made in three different steps: first, to evaluate lethal effects of both molluscicides in time, second to detect changes on the activity of three enzymatic biomarkers AChE, GST and CAT upon molluscicidal exposure, understand their modes of action in isopods, and question the use of biomarkers as early warning tools in this kind of exposures, and finally, to identify the combined effect of the two chemicals to this terrestrial isopod.

2.2 Materials and methods

2.2.1 Test organisms

The isopods used in this experiment were obtained from a laboratorial culture, maintained in a climatic chamber at 25ºC, 60% moisture content, and with a 16h: 8h light: dark photoperiod. Only adult animals (15–25 mg wet weight) with antenna were used. In the beginning of the test, no sex differentiation was done, although pregnant females were not used in the experimental procedure.

2.2.2 Test chemicals

Two molluscicides were used in the experiment: metaldehyde and methiocarb, as the commercial formulations of CARAKOL® (Impex™) containing 5% of metaldehyde and MESUROL® (Bayer™) with 4% of methiocarb, respectively. Tests were performed in LUFA 2.2 soil, commercialized by the German Institution LUFA Speyer. The soil used was characterized by the following properties: pH of 5.5, organic matter content of 3.9%, and 6% of clay, 17% of silt and 77% of sand.

2.2.3 Experimental procedure

2.2.3.1 Acute bioassay

An acute bioassay was performed to establish the sensitivity of *Porcellionides pruinosus* to the molluscicide baits through time. Twenty isopods were exposed to 5 metaldehyde baits for a period of 56 hours, and 20 isopods were exposed to 3 baits of methiocarb for 24 hours. Isopods' fitness was routinely checked and registered, and the lethal time (LT_{50}) after the beginning of exposure to the molluscicide baits was calculated. Considering the LT_{50} values calculated, several single exposure periods were chosen to evaluate the sub-lethal effects of each of the pesticides.

2.2.3.2 Single chemical exposure

Each animal was placed individually in a plastic box, with a surface area of 0.0064 m^2 containing 30 g wet soil. In this experiment we used 5 baits of metaldehyde per test-box, which gives a concentration 20 times higher than the recommended dose (39 pellets per m^2). The recommended dose for the carbamate methiocarb is 20 baits per m^2 and in the experimental procedure we tested 3 pellets per test-box, giving a concentration 30 times higher than the field recommended dose. Even though this scenario can be widely found in agriculture fields or gardens, because molluscicides application is done randomly and baits are usually found at high densities in some areas, forming agglomerates.

In this experimental setup isopods were collected 8, 16, 24 and 32 hours after metaldehyde exposure, and 1, 2, 3 and 4 hours after methiocarb exposure. Ten isopods were sampled per time of exposure. After each of these sampling times 5 isopods were collected from a control test-box, maintained under the same conditions although without any molluscicide baits. Thus, a total of 60 isopods was analysed per molluscicide. Isopods were then frozen at –80°C until the biomarker analyses were performed. In addition, several test-boxes with different exposure periods to control situations were also included in this experiment to determine if there were any shifts in the enzymatic activities throughout time.

2.2.3.3 Experimental procedure – mixture toxicity exposure

For the mixture toxicity tests, isopods were exposed to the two molluscicides in six different combinations for time of exposure. Each combination comprised 10 isopods exposed individually to 3 baits of methiocarb (Mb) and 5 baits of metaldehyde (Md), alternatively and never simultaneously (Table 2.1). In addition, several isopods were also kept in a test-box without any contamination (control conditions) as the mixture experiment went on. 5 isopods were collected after 17 hours, 5 isopods were collected after 25 hours and finally 10 isopods were collected after 34 hours. Again, after each sampling time, animals were stored at –80ºC until the biomarkers analyses were performed.

Table 2.1: Design experiment of the six combinations performed with three baits of methiocarb and five baits of metaldehyde.

Combination	First exposure period	Second exposure period
Mb1hMd16h	1h to methiocarb	16h to metaldehyde
Mb1hMd32h	1h to methiocarb	32h to metaldehyde
Md32hMb1h	32h to metaldehyde	1h to methiocarb
Md32hMb2h	32h to metaldehyde	2h to methiocarb
Mb1hMd24h	1h to methiocarb	24h to metaldehyde
Mb2hMd24h	2h to methiocarb	24h to metaldehyde

2.2.4 Preparation of the animals

For the biomarker analysis isopods were divided in two sections: head and body. The head was used for the AChE assay and the remaining body was used for GST and CAT assays. Homogenisation of the animals was made using a sonicator (KIKA Labortechnik U2005 Control™).

2.2.5 Determination of AChE activity

After sonication, samples using isopods' heads were centrifuged at 1,700g for 3 min at 4ºC. The obtained supernatant was immediately assayed for AChE activity according to the Ellman technique (1961) adapted to the microplate (Guilhermino et al. 1996). In a 96 well microplate 250 µl of the reaction solution was added to 50 µl of the sample and absorbance was recorded at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280 ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The

enzyme activity is expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

2.2.6 GST analysis

Glutathione *S*-Transferase activity was determined based on the method described by Habig et al. (1974) and adapted to microplate (Diamantino et al. 2001). We mixed 100 µL of PMS in 200 µL of a reaction solution. The reaction solution was a mixture of 4.950 ml K-Phosphate 0.1 M (pH 6.5) with 900 µL GSH 10mM, and 150 µL 1-chloro-2,4-dinitrobenzene (CDNB) 10mM, and was measured at 340 nm. The enzyme activity is expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $9.6 x10^{-3}$ M^{-1} cm^{-1}.

2.2.7 CATALASE analysis

Catalase activity was determined based on the method described by Clairborne (1985), consisting in mixing 50 µl of PMS with 500 µL H_2O_2 0.030 M, and 950 µL K-Phosphate 0.05 M (pH 7.0) and measuring the decomposition of the substrate (H_2O_2) at 240 nm. The enzyme activity is expressed as unit (U) per mg of protein. A U corresponds to a µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

2.2.8 Statistical analyses

The time after which 50% of the animals were found dead in the test-boxes following molluscicide application (LT_{50}) was calculated using a logistic equation (STATISTICA 7).

Enzymatic activities obtained in the different controls (different time periods) were compared using a one way ANOVA (SIGMA STAT 3.5.) to detect possible differences

between animals collected in different periods from the control test-boxes. If enzymatic activities in control animals were not different in any of the experiment periods, these values were pooled and used as a total mean control to compare changes in enzymatic activities of animals exposed to the molluscicides also using a one way ANOVA (SIGMA STAT 3.5.). Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal–Wallis ANOVA on Ranks was performed, followed by the Dunnett's method when significant differences were found.

2.3 Results

2.3.1 Acute bioassay

After a period of 24 h of continuous exposure to methiocarb baits all the animals in the test boxes were found dead, and a LT_{50} = 7.27 (5.38h–9.18h) was obtained (Fig. 2.1). This result clearly indicates the severe toxicity of this molluscicide to the terrestrial isopod *Porcellionides pruinosus*. The application of metaldehyde baits also resulted in an high mortality rate (Fig. 2.1) after the 56h period of exposure with a calculated LT_{50} of 50.49h (50.48–50.50h). It was observed that upon exposure, isopods detected (by smell or vision) the blue baits, and almost immediately direct themselves to the baits, walking around them and bite a small fraction of one bait. After this episode, isopods start to show effects from baits toxicity like changes in pattern mobility.

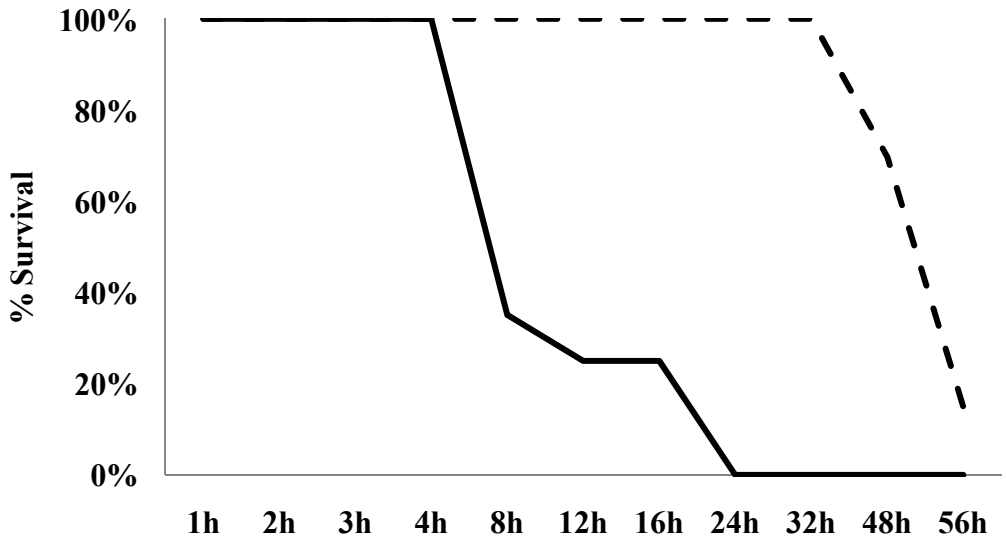

Fig. 2.1 - Percentage of survival of the isopod *Porcellionides pruinosus* exposed to three methiocarb baits (straight line) and five metaldehyde baits (dashed line) in time.

2.3.2 AChE activity

No statistical differences were found between activities observed in controls periods of metaldehyde (P=0.473), methiocarb (P=0.824) and combined experiments P=0.463).

Metaldehyde did not have any effects in the activity of AChE of the isopod *Porcellionides pruinosus.* After comparing the four exposure periods with the correspondent control values no differences were found in this enzyme activity. The results in AChE activity were homogenous during the 32 hours of exposure and no variations were found between the animals exposed (Fig. 2.2).

Methiocarb significantly inhibited AChE activity in *P. pruinosus* already after the first hour of exposure to the molluscicide baits and throughout the experimental period. In all sampling times the enzymatic activity of exposed animals was smaller than the control, with its lowest activity being observed after 4 hours of exposure (53,997 U/mg protein), showing an inhibition of 42% compared with control activity (Fig. 2.2).

In the joint toxicity experiments a significant inhibition of AChE activity was found in almost all the combinations performed ($H = 60.19$, $P \leq 0.001$), with the exception of the combination of Md32hMb1h, where the activity obtained in the exposed animal (132.81 U/mg protein) was similar to control animals. Although not expected, a significant increase in AChE activity in comparison to the control was observed in the combined effect of 32 hours of metaldehyde plus 2 hours of exposure to methiocarb (Fig. 2.2). In this combination the value calculated (281.47 U/mg protein) was more than the double observed in the control (130.50 /mg protein). This experiment was repeated twice and the pattern of response of increased AChE activity in the referred combination was consistent in all experiments made.

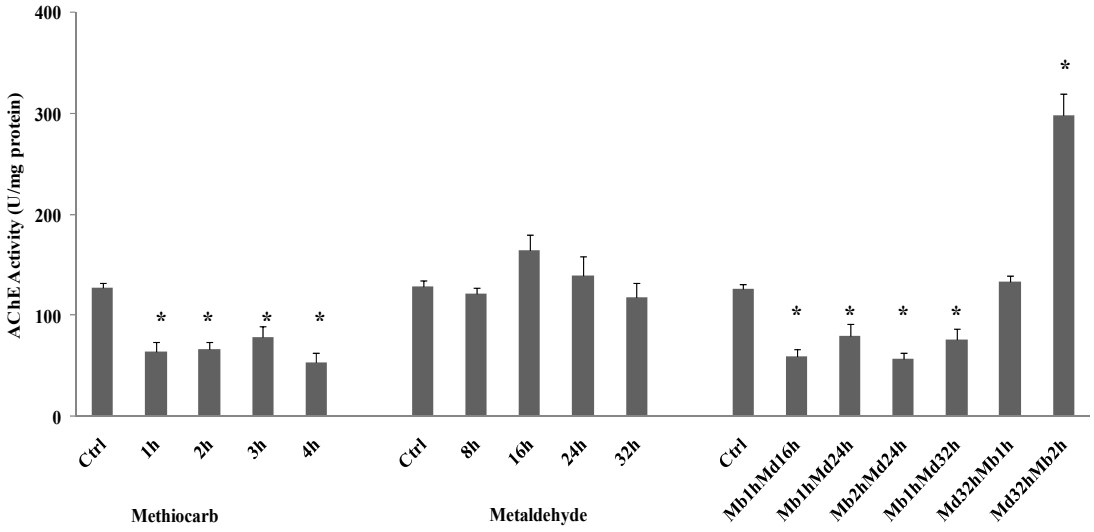

Fig. 2.2 - AChE activity in *Porcellionides pruinosus* exposed to metaldehyde, methiocarb and six different combinations of the two baits. Results are expressed as the mean value of AChE activity (U/mg protein) with associated standard error. * indicates significant differences between control and treatments ($P \leq 0.05$)

2.3.3 GST activity

No statistical differences were found between activities observed in controls periods of metaldehyde (P=0.853) methiocarb (P=0.581) and combined experiments (P=0.682).

An increase in GST activity after giving metaldehyde baits to the isopods was observed after 16h of exposure exhibiting 125% of the activity calculated for the control animals (Fig. 2.3). Extending the exposure periods to 24 and 32 h GST activity decreased. In these two experimental periods the enzymatic values calculated for 24h and 32h was 37.98 and 39.92 U/mg protein, resulting in an inhibition of 73% and 78%, respectively.

Methiocarb did not have any effects in GST activity (Fig. 2.3); values calculated for 24h and 32h and respective control periods were consistent during the experimental period without any oscillation of the calculated values of activity. In addition, the combination of the two molluscicides did not provoke any effects in GST activity, in all combinations the values were similar to those observed in the respective control (Fig. 2.3).

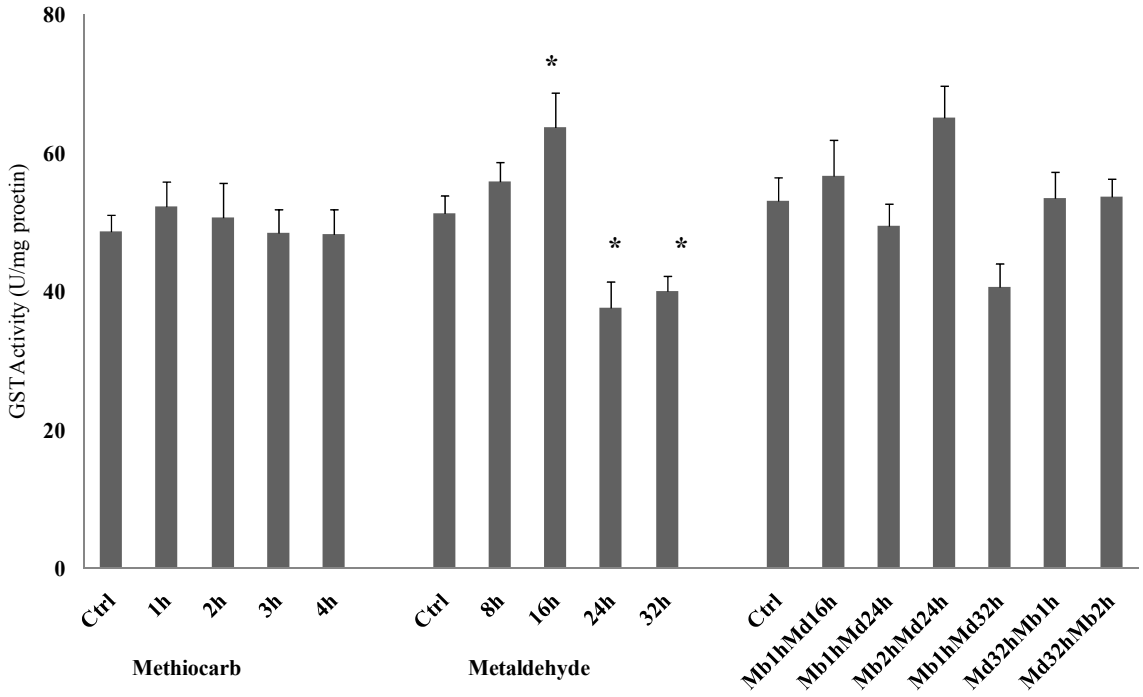

Fig. 2.3 - GST activity in *Porcellionides pruinosus* exposed to metaldehyde, methiocarb and six different combinations of the two baits. Results are expressed as the mean value of GST activity (U/mg protein) with associated standard error. * indicates significant differences between control and treatments ($P \leq 0.05$)

2.3.4 CAT activity

No statistical differences were found between activities observed in control periods of metaldehyde (P=0.506) methiocarb (P=0.122) and combined experiments (P=0.433).

The application of metaldehyde baits caused an increase in CAT activity in all exposure periods but the 16h sampling time (Fig. 2.4). This increase in CAT activity was more evident after the periods of 24h, where the mean values in exposed animals (409.05 U/mg protein) were 6 times higher than the values in the control (86.63 U/mg protein). Also in the last sampling time (32h) the mean values of the antioxidant enzyme in the animals exposed to the baits were twice the values in control situation (228.47 U/mg protein). CAT activity values were consistent along the four hours of exposure to methiocarb baits, since no variation in this enzyme activity was observed (Fig. 2.4). Although an inhibition of 71% and 63% of CAT activity was found after 2h and 3h of exposure to this molluscicide, no significant differences were found after the statistical procedure. In the mixture experiments, only two combinations (Mb1hMd24h and

Mb1hMd32h) were statistically different from the control (Fig. 2.4), showing and inhibition of 30% and 33% when compared to control mean values.

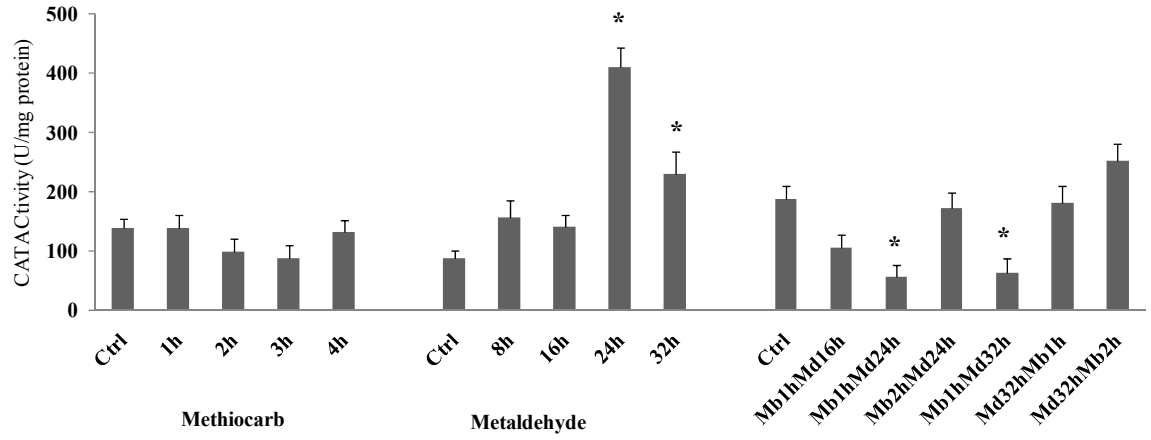

Fig. 2.4 – Catalase activity in *Porcellionides pruinosus* exposed to metaldehyde, methiocarb and six different combinations of the two baits. Results are expressed as the mean value of CAT activity (U/mg protein) with associated standard error. * indicates significant differences between control and treatments ($P \leq 0.05$)

2.4 Discussion

Methiocarb and metaldehyde caused severe lethal effects to the terrestrial isopod *Porcellionides pruinosus* in short-term exposures, although methiocarb and metaldehyde are considered non-harmful to terrestrial invertebrates, mainly due to its rapid dissipation in soil (Meredith 1996, Bieri et al. 2003). The scarce amount of time needed to observe lethal effects after bait consumption in the acute bioassay performed clearly indicates that molluscicides application represents a risk for these terrestrial invertebrates. Regarding the rapid effects that these compounds induce, a regular risk assessment procedure might not be possible as dose-effects assessments are not clear. Instead, time to death can be considered a useful approach in situations similar to this one, where short time periods (e.g. few hours) are enough to induce lethality. In addition chronic evaluation, using

sublethal concentrations will be improbable as dosages are not controlled. As explained before, it was observed that after some minutes of exposure, isopods were attracted by the baits and bitted a small portion of one of the bait, resulting is almost immediate effects (changing in mobility patterns). Thus, the dose applied in this study can not be related to the effects and will be difficult to quantify.

Metaldehyde did not have any effect in the AChE activity during the exposure period of 32 hours, since no variations in AChE levels were observed in the exposure periods sampled. This seems to indicate that metaldehyde and its metabolic secondary product aldehyde, tested in the conditions described, do not affect this enzyme activity.

Following application of the molluscicide metaldehyde some authors indicate a diminution in AChE activity in the nervous tissue of the snail *Lymnaea acuminata* (Tiwari et al., 2008). Others (Putchakayala and Ram 2000) found that although having been responsible for an excitatory effect in muscle contraction in the zebra mussel, metaldehyde did not enhance or inhibit the effects of acetylcholine, probably because the two stimulants activate different cells within the organism. The application of methiocarb strongly inhibited AChE activity being reduced after 1 hour of exposure to almost 50 % compared to the control. This drastic inhibition of AChE activity surely can be linked with the mode of action of the carbamate methiocarb, which is designed specifically to inhibit this enzyme, as it was attested by several research papers concerning the effects of this pesticide in AChE activity of various non-target species (Taylor 1996; Wellman and Heimbach 1996; Jensen 1998; Hyne and Maher 2003; Boran et al. 2007; Radwan et al. 2008). The strong decrease in AChE activity within a small exposure period is also in agreement with previous studies that illustrated the decrease in the activity of this enzyme when terrestrial isopods were exposed to organophosphate insecticides, that have a similar inhibitory influence in AChE as carbamates, even at sublethal concentrations (Fischer 1997; Ribeiro et al. 1999; Stanek et al. 2003; Engenheiro et al. 2005). The depression in AChE activity as a direct result of *Porcellionides pruinosus* ingestion of methiocarb pellets and the sensitivity of this enzyme to exposure represents a specific response and is of critical importance in the determination of the mode of action of this molluscicide to terrestrial isopods, and therefore can be considered a precise biomarker of exposure (Peakall 1999).

In the combinations where the methiocarb pellets were first given to the animals, AChE activity decreased in comparison with the control animals. In these cases, apparently the subsequent period of exposure to metaldehyde baits had no effect in the enzymatic activity, since enzyme activity was comparable with the single experiments with methiocarb tested with the same exposure period. It is noteworthy, however, to observe that in one mixture AChE activity increased in comparison to control values. The combination comprised 32 hours of exposure to metaldehyde plus 2 hours of exposure to methiocarb. In this treatment the values obtained for AChE activity were the highest of all the experiments performed and almost twice the activity registered in the single toxicity experiment with metaldehyde for 32 hours. Previous works using the fresh water snail *Lymnaea stagnalis* (Mills et al. 1990; Mills et al. 1992) described an increase in firing activity and development of paroxysmal depolarising shifts in buccal motoneuron, indicating severe alterations in the central nervous system (CNS) after application of metaldehyde. This suggests that these alterations could provide a useful model for screening the effects of molluscicides on a functional neuronal network. An induction in the AChE activity has been recorded in cytochemical experiments, in which sequence analysis revealed that AChE expressed in apoptotic cells could be considered a biomarker and a regulator of apoptosis (Zhang et al. 2002). The results seem to indicate that the application of this molluscicide has an impact on the CNS of *Porcellionides pruinosus*, and that increasing time of contact with the pellets induces an augment of AChE activity. From the above, one can hypothesize that the increase in AChE activity might be a result of intra-specific responses of the CNS of this isopod species to the application of metaldehyde baits in combination with methiocarb baits.

Following the application of metaldehyde, GST activity increased significantly after 16h of exposure and this induction might be related to the presence of the xenobiotic and subsequent activation of the natural detoxifying defence system (Van der Oost et al. 2003). After 24h and 32h of exposure, the values of GST activity decreased in comparison with the values in the control animals. It seems that after triggering GST levels, the continuous exposure to metaldehyde led to an inhibition of GST activity, which is in conformity with other studies where early induction of GST was not sustained during longer exposure periods (Ferrari et al. 2007). In adults of the isopod species *Porcellio scaber* a decreased GST activity was also found after application of the neonicotinoid

imidacloprid (Drobne et al. 2008), which is in conformity with our results. It is important to state that this induction of GST activity and subsequent decrease with time of exposure not only reveals the role of this detoxifying enzyme in the biotransformation of metaldehyde, but is a valuable contribution to understand the mode of action of this molluscicide to *Porcellionides pruinosus*.

The relatively homogenous response throughout the test period seems to point out that methiocarb does not have an effect on GST activity, since no induction or reduction in the basal levels of this enzyme was detected. This indicates that at least in the tested exposure period no activation of this xenobiotic metabolizing enzyme occurs, which also has been observed in previous works where this enzyme conjugate was proven to be insensitive to the application of carbamates (Ribera et al. 2001) and organophosphorous pesticides (Massa et al. 2008) in earthworms and lepidopters. The lack of sensitivity of GST to some classes of insecticides may be related to intrinsic and molecular properties of this enzymatic complex (Crane et al. 2002), and in some cases the time of exposure or concentration of the xenobiotic is not enough to activate this enzymatic complex.

In the binary experiments with the two molluscicides no changes in GST activity were observed, even in the combinations where metaldehyde baits were given at first, at concentrations that caused a decrease in the GST levels in single toxicity experiments. The subsequent period of exposure to methiocarb baits could be responsible for a "compensation" in the enzymatic levels, and thus the inhibitory effects are not evident in the combination experiments. This response in all combinations tested, with no variations found along the exposure periods, seems to confirm that this enzymatic conjugate can not always be used to detect an impairment caused by pesticide application, and thus results should be interpreted carefully (Hyne and Maher 2003).

Among biomarkers used in toxicological evaluation, those based on antioxidant defences, like catalase, reveal potentially impairment in an organism's fitness mediated by the formation of ROS (Bochetti et al. 2006). The increase in ROS production is known to result in an increased oxidative damage to macromolecules and alterations in critical cellular processes (Howcroft et al. 2009). Following application of metaldehyde an increase in CAT activity was observed in the first two periods of exposure, with a significant increase in the 24h and 32h exposure periods. This result is in conformity with

previous studies that also observed a significant increase in CAT activity in terrestrial snails after the administration of metaldehyde (el-Wakil and Radwan 1991), and might be indicative of a defence mechanism towards cellular damage occurring in the organism as a consequence of ROS formation.

CAT activity did not have statistical differences in the four sampling times of methiocarb exposure when compared with control values, although a decrease was observed after 2h and 3h of exposure to the carbamate. A decrease in CAT activity upon pesticide exposure has been described for other isopod species, like *Porcellio scaber* (Jemec et al. 2008), mussels and daphnids (Khessiba et al. 2005, Jemec et al. 2007). This non-activation of CAT activity in this isopod species might also be related to the results obtained in the enzyme GST, which was also not induced by the exposure to methiocarb and the fact that the short period of exposure to the carbamate was not sufficient to induce changes, since it is known that carbamate pesticides do cause oxidative stress and are responsible for the activation of CAT (Maran et al. 2009).

An inhibitory pattern of CAT activity was observed in the combinations experiment where the animals first received methiocarb baits. In only two of the sampling times the decrease on CAT activity was statically significant (Mb1hMd24h, Mb1hMd32h), and the following period of exposure to metaldehyde baits was not enough to reverse the effects obtained in these combinations. The decreasing activity of CAT observed in the single exposure to methiocarb seems to be confirmed in this combined experiment, since statistical differences from the control were observed. This could be a consequence of a longer period of exposure of the animals to the baits in the combined experiment in comparison with the single administration of methiocarb. In the combinations where metaldehyde baits were given at first no differences in CAT activity were found in comparison with the enzymatic levels observed in the control. These results were not in accordance with the extreme increase in CAT activity found in single toxicity experiments with metaldehyde, but probably the subsequent exposure to methiocarb baits could be responsible for the mitigation of an increased CAT activity during the exposure to metaldehyde baits.

2.5 Conclusions

The LT50 values found in the single exposures to both molluscicides were very low, especially in the case of the carbamate methiocarb. This clearly indicates the severity of the exposure to these baits by terrestrial isopods, since even a short exposure to the baits redounded to an extremely high mortality rate. So, it seems pertinent to take this into account in risk assessment schemes for molluscicides application in agricultural fields or gardens, and its impact on macrofauna like terrestrial isopods. As expected, due to the specificity of this enzyme AChE activity seems to be an accurate biomarker of exposure to the carbamate methiocarb. The two enzymes GST and CAT, involved in protecting cellular damages from oxidative stress, confirmed also to be useful in assessing metaldehyde exposure. The use of several biomarkers was a suitable tool to understand the mode of action of these two molluscicides in this isopod species.

References

Bailey SER (2002) Molluscicidal baits for control of terrestrial gastropods. In: Barker, GM (ed), Molluscs as crop pests, CABI Publishing, Wallingford, U.K., pp 33–54

Bieri, M (2003) The environmental profile of metaldehyde. In: Henderson IF (ed) Slugs and Snails in World Agriculture, British Crop Protection Council, Symposium Proceedings 80, pp 255–260

Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere 65: 913–921

Boran M, Altinok U, Çapkin E, Karaçam H, Biçer V (2007) Acute toxicity of carbaryl, methiocarb, and carbosulfan to the rainbow trout (*Oncorhynchus mykiss*) and Guppy (*Poecilia reticulata*). Turk J Vet Anim Sci 31:39–45

Brown PJ, Long SM, Spurgeon DJ, Svendsen C, Hankard PK (2004) Toxicological and biochemical responses of the earthworm *Lumbricus rubellus* to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon. Chemosphere 57:1675–168

Clairborne A (1985). Catalase activity. In: Greenwald R (ed) Handbook of methods for oxygen radical research, CRC Press, Boca Raton, Florida, pp 283–284

Crane M, Sildanchandra W, Kheir R, Callaghan C (2002) Relationship between biomarker activity and developmental endpoints in *Chironomus riparius* Meigen exposed to an organophosphate insecticide. Ecotox Environ Saf 53(3):361–369

Diamantino TC, Almeida E, Soares AVMV, Guilhermino L (2001) Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* straus. Chemosphere 45:553–560

Drobne D, Blažič M, Van Gestel CAM, Lešer V, Zidar P, Jemec A, Trebše P (2008) Toxicity of imidacloprid to the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). Chemosphere 71:1326–1334

Ellman GL, Courtney KD, Andres VJ, Featherstone R (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmac 7:88–95

El-Wakil HB, Radwan MA (1991) Biochemical studies on the terrestrial snails *Eobania vermiculata* (Müller) treated with some pesticides. J Environ Sci Health Part B 26(5–6):479–489

Engenheiro EL, Hankard PK, Sousa JP, Lemos MF, Weeks JM, Soares AMVM (2005) Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environ Toxicol Chem 24(3):603–609

Ferrari A, Venturino A, D´Angelo AMP (2007) Effects of carbaryl and azinphos methyl on juvenile rainbow trout (*Oncorhynchus myskiss*) detoxifying enzymes. Pest Biochem Physiol 88:134–142

Fischer E, Farkas S, Hornung E, Past T (1997) Sublethal effects of an organophosphorus insecticide dimethoate on the isopod *Porcellio scaber*. Comp Biochem Physiol Part C: Toxicol Pharmacol 116(2):161–166

Fulton MH, Key PB (2001) Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ Toxicol Chem 20(1):37–45

Guilhermino L, Lopes MC, Carvalho AP, Soares AMVM (1996) Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32:727–738

Habig, H, Pabst, MJ Jakoby WB (1974): Glutathione *S*-transferase, the first enzymatic step in mercapturic acid formation. J Biol Chem 249: 7130–7139.

Hayes JD, Pulford DJ (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol 30:445–600

Henderson I, Triebskorn R (2002) Chemical control of terrestrial gastropods. In: Barker GM (ed) Molluscs as crop pests, CABI Publishing, Wallingford, U.K., pp 1–31

Hyne RV, Maher WA (2003) Invertebrate biomarkers: links to toxicosis that predict population decline. Ecotoxicol Environ Saf 54:366–374

Howcroft CF, Amorim MJB, Gravato C, Guilhermino L, Soares AMVM (2009) Effects of natural and chemical stressors on *Enchytraeus albidus*: Can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? Environ Int 35:318–324

Jemec A, Drobne D, Tišler T, Trebše P, Ros M, Sepčić K (2007) The applicability of acetylcholinesterase and glutathione S-transferase in *Daphnia magna* toxicity test. Comp Biochem Physiol Part C: Toxicol Pharmacol 144:303–309

Jemec A, Drobne D, Remskar M, Sepčić K, Tisler T (2008) Effects of ingested nano-sized titanium dioxide on terrestrial isopods (*Porcellio scaber*). Environ Toxicol Chem 27(9):1904–1914

Jensen S (1998) Acetylcholinesterase Activity Associated with Methiocarb Resistance in a Strain of Western Flower Thrips, *Frankliniella occidentalis* (Pergande). Pestic Biochem Physiol 61:191–200

Khessiba A, Roméo M, Aïssa P (2005) Effects of some environmental parameters on catalase activity measured in the mussel (*Mytilus galloprovincialis*) exposed to lindane. Environ Poll 133:275–281

Köhler HR, Hiittenrauch K, Berkus M, Gräff S, Alberti G (1996) Cellular hepatopancreatic reactions in *Porcellio scaber* (Isopoda) as biomarkers for the evaluation of heavy metal toxicity in soils. Appl Soil Ecol 3(1):1–15

Köhler HR, Knödler C, Zanger M (1999) Divergent Kinetics of hsp70 Induction in *Oniscus asellus* (Isopoda) in Response to Four Environmentally Relevant Organic Chemicals (B[a]P, PCB52, g-HCH, PCP): Suitability and Limits of a Biomarker. Arch Environ Contam Toxicol 36:179–185

Loureiro S, Sousa JP, Nogueira AJA, Soares AMVM (2002) Assimilation efficiency and toxicokinetics of 14C-lindane in the terrestrial isopod *Porcellionides pruinosus*: the role of isopods in degradation of persistent soil pollutants. Ecotoxicol 11:481–490

Loureiro S, Soares AMV, Nogueira AJA (2005) Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ Poll 138:121–131

Loureiro, S, Amorim, MJB, Campos, B, Rodrigues, SMG, Soares, AMVM (2009). Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ Pollut 157 (2): 625-632.

Maran E, Fernández M, Barbieri P, Font G, Ruiz MJ (2009) Effects of four carbamate compounds on antioxidant parameters. Ecotox Environ Saf 72:922–930

Massa R, Blevins S, Chao SL (2008) Role of acetylcholinesterase and glutathione S-transferase following exposure to nicosulfuron and diazinon in *Helicoverpa zea*. Ecotoxicol Environ Saf 71:230–235

Meredith RH (1996) Testing bait treatments for slug control. In Henderson IF (ed) Slug and Snail Pests in Agriculture, British Crop Protection Council, Farnham, pp 157–164

Mills JD, McCrohan CA, Bailey, SER, Wedgwood MA (1990) Effects of metaldehyde and acetaldehyde on feeding responses and neuronal activity in the snail *Lymnaea stagnalis*. Pestic Sci 28:189–199

Mills JD, McCrohan CR, Bailey SER (1992) Electrophysiological responses to metaldehyde in neurones of the feeding circuitry of the snail *Lymnaea stagnalis*. Pestic Biochem Physiol 42(1–2):35–42

Peakall DB (1999) The use of biomarkers in hazard assessment. In: Peakall DB, Colin HW, Migula P (eds.) Biomarkers: a Pragmatic Basis for Remediation of Severe Pollution in Eastern Europe, Kluwer Academic Publishers, Dordrecht, Netherlands, pp 123–133

Putachakayala SM, Ram JL (2000) Toxic and excitatory effects of the molluscicide metaldehyde on the biofouling bivalve *Dreissena polymorpha* Pallas. Pest Manag Sci 56:39–42

Radwan MA, Essawy AE, Abdelmeguied NE, Hamed SS, Ahmed AE (2008) Biochemical and histochemical studies on the digestive gland of *Eobania vermiculata* snails treated with carbamate pesticides. Pestic Biochem Physiol 90:154–167

Ribeiro S, Guilhermino L, Sousa JP, Soares AMVM (1999) Novel Bioassay based on Acetylcholinesterase and Lactate Dehydrogenase activities to evaluate the toxicity of chemicals to soil isopods. Ecotoxicol Environ Saf 44:287–293

Ribera D, Narbonne JF, Arnaud C, Saint-Denis M (2001) Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil, effects of carbaryl. Soil Biol Biochem 33:1123–1130

Roberts AP, Oris JT (2004) Multiple biomarker response in rainbow trout during exposure to hexavalent chromium. Comp Biochem Physiol Part C: Toxicol Pharmacol 138:221–228

Schlenk D (1999) Necessity of defining biomarkers for use in ecological risk assessments. Mar Pollut Bull 39(1–12):48–53

Schreck E, Geret F, Gontier L,Treilhou M (2008) Neurotoxic effect and metabolic responses induced by a mixture of six pesticides on the earthworm *Aporrectodea caliginosa nocturna*. Chemosphere 71:1832–1839

Stanek K, Gabrijelcic E, Drobne D, Trebše P (2003) Inhibition of acetylcholinesterase activity in the terrestrial isopod *Porcellio scaber* as a biomarker of organophosphorous compounds in food. Arh Hig Rada Toksikol 54:183–188

Stanek K, Drobne D, Trebše P (2006) Linkage of biomarkers along levels of biological complexity in juvenile and adult diazinon fed terrestrial isopod (*Porcellio scaber*, Isopoda, crustacea). Chemosphere 64:1745–1752

Stroomberg GJ, de Knecht JA, Ariese F, van Gestel CAM, Velthorst NH (1999) Pyrene metabolites in the hepatopancreas and gut of the isopod *Porcellio scaber*, a new biomarker for polycyclic aromatic hydrocarbon exposure in terrestrial ecosystems. Environ Toxicol Chem 18(10):2217–2224

Taylor P (1996) Agents acting at the neuromuscular junction and autonomic ganglia. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Goodman GA (eds) The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, pp 177–198

Tiwari F, Singh K, Singh DK (2008) Enzyme inhibition by different bait formulations in the nervous system of the snail *Lymnaea acuminate*. In: Joshi BD, Joshi PC, Joshi N (eds) Environmental Pollution and Toxicology, A.P.H. Publishing Corporation, New Dehli. pp 115–128

Triebskorn R, Christensen K, Heim I (1998) Effects of orally and dermally applied metaldehyde on mucus cells of slugs (*Deroceras reticulatum*) depending on temperature and duration of exposure. J Molluscan Stud 64:467–487

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Wellmann P, Heimbach F (1996) The effects of methiocarb slug pellets on the earthworm *Lumbricus terrestris* in a laboratory test. In: Henderson IF (ed) Slug and Snail Pests in Agriculture, British Crop Protection Council: Farnham, pp 181–188

Zhang XJ, Yang L, Zhao Q, Caen JP, He HY, Jin QH, Guo LH, Alemany M, Zhang LY, Shi YF (2002) Induction of acetylcholinesterase expression during apoptosis in various cell types. Cell Death Differ 9:790–800

Zimmer M (2002) Nutrition in terrestrial isopods (Isopoda:Oniscidea): an evolutionary-ecological approach. Biol Rev Camb Philos Soc 77:455–493

3. JOINT EFFECTS OF THREE PLANT PROTECTION PRODUCTS TO THE TERRESTRIAL ISOPOD *PORCELLIONIDES PRUINOSUS* AND THE COLLEMBOLAN *FOLSOMIA CANDIDA*

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Abstract

The effects of simultaneous application of plant protection products are of concern since the uses of different products pose an additional risk to non-target soil organisms. The effects of binary combinations of dimethoate, glyphosate and spirodiclofen, an insecticide an herbicide and an acaricide, on the avoidance behaviour of the terrestrial isopod *Porcellionides pruinosus* and the reproductive effort of *Folsomia candida* were assessed using the two reference models of concentration addition (CA) and independent action (IA). Results of single exposure to the three pesticides indicated a clear dose related avoidance response of the isopods in the highest concentrations tested of the three as well as a strong decrease in collembolan adult survival and concomitant number of juveniles produced. In the combined experiments, antagonism was found in 7 out of the 12 combinations, 4 combinations followed the reference models, and only in one combination synergism was detected (lower doses of glyphosate and spirodiclofen applied to *P. pruinosus*). In conclusion, it seems that mixing and applying these products, at the recommended field application rate, does not lead to enhanced toxicity, hence limited risk is associated with the joint application of these pesticides.

3.1. Introduction

Chemical risk evaluation is based on the assessment of the effects of single chemicals to single test-species although in real scenarios organisms are exposed to mixtures of chemicals (Baas et al, 2007). In agricultural fields, for example, several pesticides are applied at the same time or in consecutive days, which could pose an additional risk to non-target organisms (Junghans et al, 2006). Therefore it seems pertinent to evaluate possible detrimental effects that may arose from the combined application of pesticides to non-target edaphic organisms (Larsen et al, 2003).

Two different concepts, imported from pharmacological studies, are used in the ecotoxicological assessment of chemical mixtures: concentration addition (CA), first described by Loewe and Muischneck (1926), and independent action (IA), described by Bliss (1939). The main difference among these two concepts is related with chemicals mode of action, since CA is used for similarly acting substances whereas IA is used for dissimilarly acting substances, although both models assume that no interaction between the two substances in the mixture takes place (Hewlett and Plackett, 1959). Both conceptual models (CA and IA) were fitted to the isopods avoidance behaviour data and collembolan reproductive output data gathered from the three binary mixtures performed.

The strategy followed in this study was based on the fact that the specific molecular mode of action of these three pesticides to the isopod *Porcellionides pruinosus* and *Folsomia candida* were unknown, thus in the absence of a clear and precise knowledge of the mechanism of toxicity that one chemical may exert to the target-organism, CA and IA can be used as equally valid reference models (Gomez-Eyles et al., 2009). In 2005, Jonker et al. described the MixTox model as a tool that can be used to derive patterns of response of binary mixtures. As a first approach this tool fit ecotoxicity data to the conceptual models (the CA or the IA) and then evaluated if there are any deviations for synergism/antagonism or dose level or ratio dependencies (i.e. depending on low or high doses, or dependent on the ratio of the chemicals in the mixture, respectively).

In soil ecosystems terrestrial isopods can be found in the soil surface layer and are responsible for the decomposition of litter contributing decisively for nutrient recycling in edaphic ecosystems (Zimmer, 2002). Their importance in influencing microbial

respiration as well in increasing the availability of macronutrients in the upper soil layer has been well established (Kautz and Topp, 2000). So, the evaluation of possible impairments in the isopods populations due to applications of pesticides is of critical importance, considering the essential role in soil ecosystems maintenance that these organisms have (Loureiro et al., 2005).

The springtail *Folsomia candida* is a useful indicator organism because it has a short life cycle, is present in high densities in terrestrial ecosystems and has a widespread distribution through all Europe (Skovlund et al., 2008) and can give an earlier indication of ecosystem disturbance (Jänsch et al., 2005). This species is parthenogenetic, easy to culture and has a relatively short generation time, so it is possible to study different individual and population parameters in one experiment (Greenslade and Vaughan, 2003). It contributes to the decomposition processes in soil by grazing on bacteria and fungi and breaking down organic matter (Petersen, 2002) so it is important to understand if agricultural practices may affect their population levels (Eaton, 2006).

Several tests have been standardized and are available to evaluate chemical exposure as the earthworms and springtails avoidance behaviour standardized tests (ISO, 2008; ISO, 2010). Avoidance behaviour, first applied to evaluate earthworm behaviour in escaping from contaminated soils (Yeardley et al, 1996), have been used to evaluate the effects of pollutants to several soil organisms (Loureiro et al, 2009; Amorim et al, 2008; Natal-da-Luz et al, 2008; Owojori and Reinecke, 2009). These tests are nowadays considered a good tool to screen soil contamination since they are cheap, quick and easy to perform and furthermore are sensitive to a broad spectrum of contaminants (Römbke, 2008). In conclusion, avoidance behaviour represents a robust endpoint to evaluate and predict the effects of environmental contamination in soil organisms (Loureiro et al, 2005; Aldaya et al, 2006).

Among the several parameters studied in ecotoxicological tests, the evaluation of the reproduction output of soil organisms is considered to be an effective and ecological relevant parameter to assess pesticide effects in terrestrial ecosystems. Thus, to assess the effects of pesticides in soil organisms standardized ecotoxicological laboratory tests mainly focused on parameters like survival and/or reproduction are available (e.g. ISO, 1999).

The herbicide glyphosate inhibits the biosynthesis of aromatic amino acids, and deregulates the shikimate pathway which leads to general metabolic disruption (Reddy et al, 2008). The insecticide dimethoate is one of the most commonly applied insecticides in agricultural fields, which acts trough the inhibition of cholinesterase enzyme activity (Martikainen, 1996). Spirodiclofen is a selective, non-systemic acaricide from the novel class of tetronic acid derivatives, which interferes with lipid biosynthesis by inhibiting acetyl-Coa carboxylase (Nauen, 2005; Wachendorff et al, 2002). The three plant protection products (PPPs) were chosen based on their different modes of action, but also due to the market share that dimethoate and glyphosate have in Europe as the most sold pesticides and spirodiclofen, which has been recently introduced in Portugal (Vieira, 2009).

Considering the possible and real scenarios of fields contaminated with PPPs cocktails and the available tools to evaluate their potential effects on key-species populations, the purposes of this study were: firstly, to determine the effects of three commonly used PPPs on the avoidance response pattern of *Porcellionides pruinosus* and in the reproductive output of *Folsomia candida*; secondly to predict the response patterns for mixture exposures using the CA and IA conceptual models for the two test-species.

3.2 Materials and Methods

3.2.1 Test organisms

The isopods used in these experiments were obtained from a laboratorial culture, where they are maintained in a climatic chamber at 25ºC, 60% moisture content, and with a 16h: 8h light: dark photoperiod. Only adult animals (15-25 mg wet weight) were used. No sex differentiation was done, although pregnant females were not chosen to the experimental procedure.

The collembolan were obtained from a laboratory culture, maintained at a constant regime of 16h light, 8h dark and a constant temperature of 17 ± 2ºC. The springtails were cultured in plastic boxes lined with a mixture of plaster of Paris and activated charcoal in a

ratio of 10:1. On a weekly basis granulated dry yeast was added as food in small amounts to avoid spoilage by fungi.

3.2.2 Test Chemicals and Test Soil

Three pesticides were used in the experimental procedure as commercial formulations: the post-emergence herbicide glyphosate (ROUNDUP® with 360 g AI/L, and which contains glyphosate-isopropylammonium (45%), surfactant (16%) and water (42.5%)), the organophosphorous insecticide dimethoate (AGROR® with 400 g AI/L and which contains dimethoate (40 %), ciclohexanone (28.4%), nonylphenol ethoxylate (2.2%), petroleum naphta (26.1%) and calcium alkyl benzene sulphonate inpropil 2-ol (0.4%)) and the acaricide spirodiclofen (ENVIDOR® with 240 g AI/L which contains spirodiclofen (23.3%), ethoxylated polyarylphenol (1-22.5%) and glycerine (> 1%)). The nominal concentrations used for glyphosate ranged from 0.5 to 54.5 mg/Kg dry soil in the avoidance experiment and between 0.1 and 2 mg/Kg dry soil in the reproduction test; for dimethoate the nominal concentration used ranged from 0.2 and 80 mg/Kg dry soil in the avoidance experiment and between 0.1 and 2 mg/Kg dry soil in the reproduction experiment; for spirodiclofen the nominal concentration used ranged from 0.04 and 2.7 mg/Kg dry soil in the avoidance and reproduction tests.

All tests were performed with LUFA 2.2 soil, commercialized by the German Institution LUFA Speyer. The properties of this soil include a pH=5.8, organic matter=3.9%, texture= 6% clay; 17% silt and 77% sand.

3.2.3 Single exposure procedure with *Porcellionides pruinosus*

The avoidance tests conducted with *Porcellionides pruinosus* were performed based on a methodology proposed by Loureiro et al (2005), consisting in exposing 10 isopods in a rectangular plastic box (14,3cm x 9,3 cm x 4,7 cm height) divided in two sections, one with the control soil and the other with the test soil (contaminated). Five concentrations of each pesticide plus the double control (both sides with control soil) were tested using 3 replicates each, in a total of 18 test boxes per pesticide (Fig. 3.1). The

volume of pesticides needed was added to distilled water and spiked in the soil (adjusted to 60% of the WHC). The pH was measured in the beginning and end of the test, and no significant changes in pH values were observed in any of the concentrations tested (pH values varied in a 5% interval). After 24 and 48 hours the number of animals in each side of the test-box was counted and mortality was registered. The percentage of avoidance was calculated using the formula $A = \frac{C - T}{N} \times 100$ (ISO, 2008), where A is the percentage of avoidance, C is the number of animal in control soil, T is the number of isopods in test (contaminated) soil and N is the total number of organisms. All calculations done were based in the nominal concentrations. The values of percentage of avoidance were used to calculate the AC_{50} in the several single exposures, and subsequently to design the binary mixture combinations.

3.2.4 Single exposure procedure with *Folsomia candida*

The experimental procedure for the reproduction test with the springtail *Folsomia candida* was performed accordingly to the ISO 11267 protocol (ISO, 1999). In the beginning of each experiment, 30 g of sieved soil (dry weight; 5mm mesh) was moistured to 60% of its water holding capacity. Five concentrations of each pesticide plus the control were tested using 5 replicates each, in a total of 30 test boxes per pesticide. Afterwards, 10 synchronised springtails (10-12 days old) were introduced in each of the five replicates. In the beginning of the test and after a 14 days' period, approximately 2 mg of dry yeast was added to all test containers. Twice a week the tests containers were opened to allow aeration, and water was added when necessary. After 28 days, the content of each test vessel was carefully transferred into larger vessels and filled up with water and some drops of blue ink. After moderate stirring, adults and juveniles floating in the water surface were photographed and counted using the image analysis software provided by Sigma Scan. The pH was measured in the beginning and end of the test, and no significant changes in pH values were observed in any of the concentrations tested (pH values varied in a 5% interval).

3.2.5 Mixture exposure

In the mixture experiments twenty-three binary combinations, based on the AC_{50} and EC_{50} values found in the previous single exposure experiments, were made simultaneously with five single concentrations of each pesticide and one control, without replication, in a total of 34 test-boxes for each binary experiment (Fig. 3.3 and Fig. 3.4). The range of toxic units (1 TU = EC_{50} or AC_{50} of the pesticide) in the binary experiments went from 0.375 to a maximum of 3 (see Fig. 3.1 and Fig. 3.2 for further details). The TU of each pesticide was never higher than 2 in any of the binary combinations made. In the mixture experiments, the number of replicates per combination was reduced to one, so that more combinations could be tested and thus a wider set of points along the response surface could be obtained (Ferreira et al., 2008). As a consequence the power of the analysis increased since the analysis performed lays on regression models and differences calculated between the data obtained and the modeled values (Jonker et al., 2005).

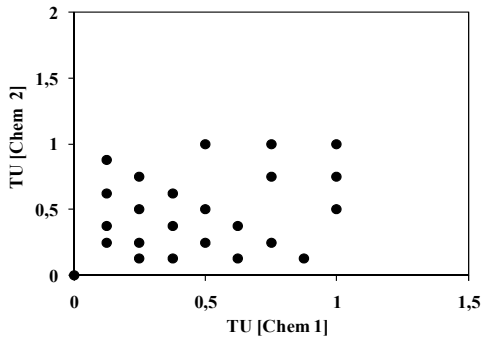

Fig. 3.1 - Experimental design of the binary combinations performed in the reproduction tests with *Folsomia candida* based on the toxic units (1 TU = EC_{50}) of glyphosate, dimethoate and spirodiclofen obtained in the single exposure tests. Data for [Chem 1] vs [Chem 2] refers to glyphosate vs dimethoate; glyphosate vs spirodiclofen; dimethoate vs spirodiclofen.

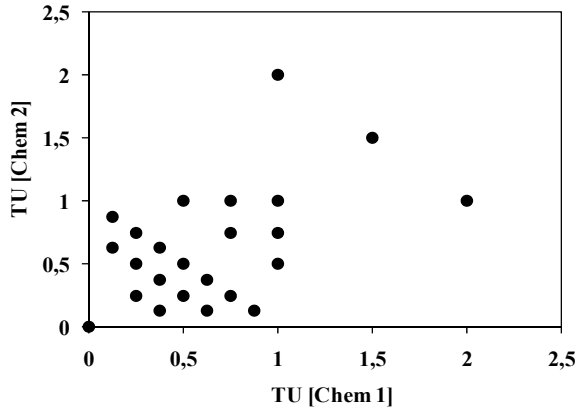

Fig. 3.2 - Experimental design of the binary combinations performed in the avoidance tests with *Porcellionides pruinosus* based on the toxic units (1 TU = EC_{50}) of glyphosate, dimethoate and spirodiclofen obtained in the single exposure tests. Data for [Chem 1] vs [Chem 2] refers to glyphosate vs dimethoate; glyphosate vs spirodiclofen; dimethoate vs spirodiclofen.

3.2.6. Statistical analysis

For the avoidance behaviour tests the concentration causing an avoidance percentage of 50% in exposed animals (AC_{50}) for each pesticide was calculated using the Probit regression scheme (MINITAB 13), assuming that when 50% of the animals are in each section of the box, no avoidance effect is observed (A=0).

For the reproduction test, differences between the number of juveniles produced by collembolans in the control and the soil treatments were analysed using a one-way ANOVA, followed by *post-hoc* Dunnett´s test (α<0.05). EC_{50} values were determined using appropriate (i.e. the best fit) non-linear models. The concentration responsible for a decrease in 50% of adult collembolans that survived the exposure period (LC_{50}) was calculated following the same procedure. For all the analysis mentioned above, STATISTICA 7.0 software provided by StatSoft Inc.,was used.

The tool used to analyze and compare the data in the present study was the MIXTOX model (Jonker et al, 2005), which allow us to fit data to both reference models (CA and IA), thus comparing the observed toxicity and the expected toxicity of the pesticides and also to calculate possible deviations from the two reference models. These

deviations are given by quantitative parameters (*a* and *b*) that can express a higher effect than expected (synergism) or a smaller effect than expected (antagonism) by the reference models (see Table 3.1 for further information). Two more complex deviations can also be obtained, depending on the level of the pesticides in the mixtures (dose-level dependence deviation) or on the composition of the mixture (dose-ratio dependence deviation).

Table 3.1: Interpretation of additional parameters (a and b) that define the functional form of deviation pattern from the reference models concentration addition (CA) and independent action (IA); adapted from Jonker et al. (2005).

Deviation Pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
synergism/antagonism	**a>0:** antagonism		
(S/A)	**a<0:** synergism		
Dose-ratio dependent (DR)	**a>0:** antagonism except for those mixture ratios where negative b value indicate synergism	b_i**>0:** antagonism where the toxicity of the mixture is caused mainly by toxicant *i*	
	a<0: synergism except for those mixture ratios where positive b value indicate antagonism	b_i**<0:** synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	**a>0:** antagonism low dose level and synergism high dose level	b_{DL}**>1:** change at lower EC50 level	b_{DL}**>2:** change at lower EC50 level
		b_{DL}**=1:** change at EC50 level	b_{DL}**=2:** change at EC50 level
	a<0: synergism low dose level and antagonism high dose level	**0<**b_{DL}**<1:** change at higher EC50 level	**1<**b_{DL}**<2:** change at higher EC50 level
		b_{DL}**<1:** No change but the magnitude of S/A is DL dependent	b_{DL}**<1:** No change but the magnitude of S/A is effect level dependent

3.3 Results

3.3.1 Single exposure procedure – *Porcellionides pruinosus*

In the double control the animal were randomly distributed through both sides of the test-box, showing no preference for one side of the box in detriment of the other side. In two replicates of glyphosate and spirodiclofen one isopod was found dead after the 48h period. In the mixtures tested no mortality was observed.

The exposure to glyphosate resulted in a clear avoidance response in the higher concentrations of the herbicide (73% avoidance at 17.4 mg/Kg) although a small decrease in the degree of avoidance response was reflected in the highest concentration (Fig. 3.3 and Table 3.2). Dimethoate caused a clear avoidance response by the isopods (Fig. 3.3), and in the two highest concentrations tested (40 and 80 mg/kg) the percentage of animals in the control soil were respectively 87% and 93% (Fig. 3.3 and Table 3.2). A strong avoidance effect was observed after the exposure of *P. pruinosus* to spirodiclofen (Fig. 3.3 and Table 3.2). Although in the concentration 0.27 mg/Kg more isopods were found in the contaminated side, when doses of spirodiclofen increased a clear avoidance response pattern was observed.

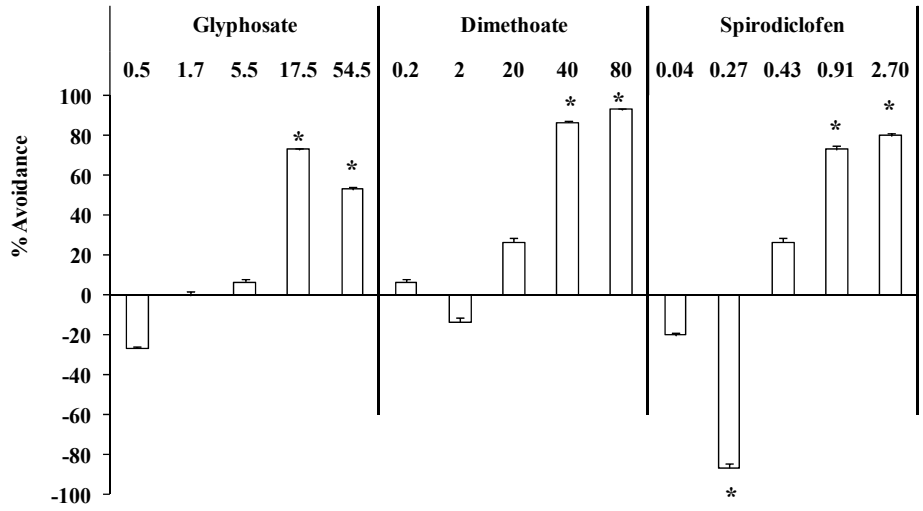

Fig. 3.3 - Avoidance response of *Porcellionides pruinosus* in LUFA 2.2 soil after application of glyphosate, dimethoate and spirodiclofen (mean net response with standard error bars). All units (nominal values) are in mg AI/Kg dry soil. (* indicates statistical differences for offspring production (Dunnett´s method, $p<0.05$)).

Table 3.2: AC_{50} values (mg/Kg dry soil) and 95% confidence intervals (CI) for the effect of single exposure pesticide on the avoidance behaviour of *Porcellionides pruinosus* exposed for 48h on LUFA 2.2 soil. Values are derived from single chemical exposure in tests run simultaneously with the mixture exposure.

Pesticide	AC_{50} (95% CI)
Glyphosate	39.7 (34.7 - 46.2) (single exposure experiment)
	16.8 (12.9 - 21.1) (mixture experiment with dimethoate)
	42.9 (39.7 - 47.1) (mixture experiment with spirodiclofen)
Dimethoate	31.5 (28.4 - 34.9) (single exposure experiment)
	31.3 (23.4 - 39.8) (mixture experiment with glyphosate)
	43.1 (37.0-50.2) (mixture experiment with spirodiclofen)
Spirodiclofen	0.9 (0.7 - 1.1) (single exposure experiment)
	1.5 (1.0 - 2.1) (mixture experiment with dimethoate)
	1.9 (1.7 - 2.1) (mixture experiment with glyphosate)

3.3.1.2 Single exposure procedure – *Folsomia candida*

The number of adult collembolans that survived the experimental procedure diminished with increasing dosages of glyphosate. This exposure resulted also in a clear reduction in the number of juveniles produced (Fig. 3.4). In the two highest concentrations of this herbicide a reduction in 86% and 95% in offspring production was observed. The LC_{50} calculated for glyphosate was 6 times higher than the value for the EC_{50} (Table 3.2). In the highest concentration tested a 98% reduction in the number of juveniles produced was observed due to the high impact in the survival rate of collembolan exposed to dimethoate The LC_{50} calculated for dimethoate was four fold the value of the EC_{50} (Table 3.2). The exposure of *F. candida* to spirodiclofen resulted in a decrease in both survival and reproduction rates with increasing dosages of this insecticide (Fig. 3.4). In the highest concentration of this insecticide there was a reduction in 100% of the adults that survived the test period. The LC_{50} calculated for spirodiclofen was similar to the one obtained in the EC_{50} calculation (Table 3.2).

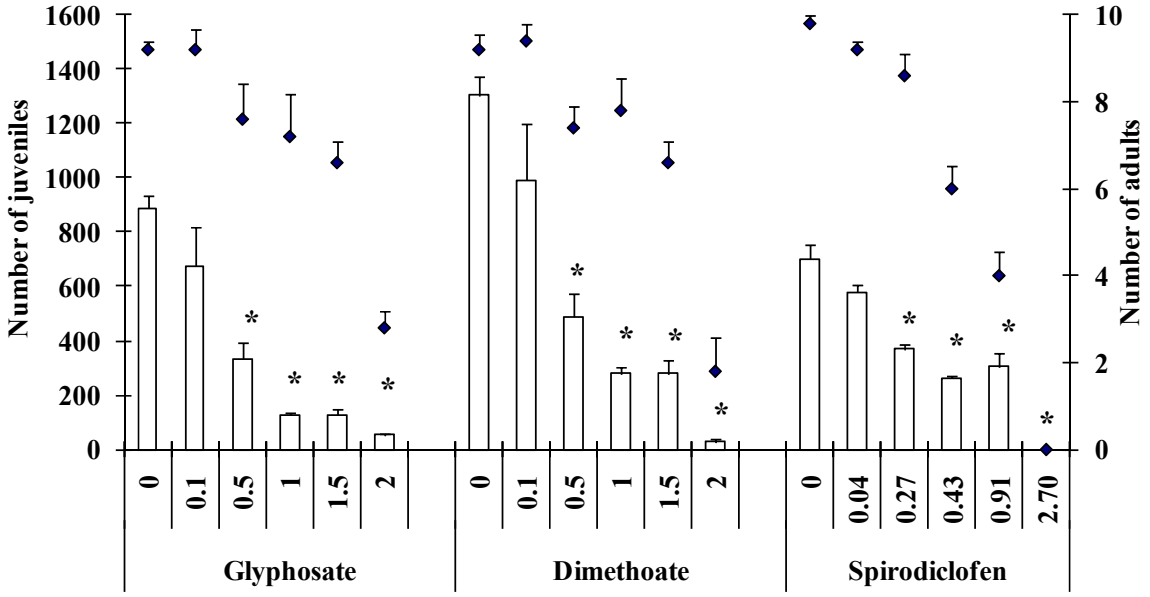

Fig. 3.4 - Reproductive output and effects on survival on *Folsomia candida* (mean number of juveniles + standard error bars) exposed to glyphosate, dimethoate and spirodiclofen spiked in the LUFA 2.2 soil. White bars indicate data for juvenile production (mean values and st. error) and black dots indicate adult survival (mean values and st. error) after the 28 d exposure. All units (nominal values) in mg AI/Kg dry soil. (* indicates statistical differences for offspring production (Dunnett´s method, $p<0.05$)).

Table 3.3: EC_{50} values (mg/Kg dry soil) and 95% confidence intervals (CI) for the effects of single exposure pesticides on the reproductive output of *Folsomia candida* exposed for 28d on LUFA 2.2 soil. Values are derived from single chemical exposure in tests run simultaneously with the mixture exposure.

Pesticide	EC_{50} (95% CI)
Glyphosate	0.33 (0.18-0.48) (single exposure experiment)
	0.15 (0.02-0.28) (mixture experiment with dimethoate)
	0.1 (0.07-0.14) (mixture experiment with spirodiclofen)
Dimethoate	0.37 (0.19-0.54) (single exposure experiment)
	0.05 (0.03-0.07) (mixture experiment with glyphosate)
	0.15 (0.08-0.21) (mixture experiment with spirodiclofen)
Spirodiclofen	0.65 (0.37-0.96) (single exposure experiment)
	0.27 (0.15-0.39) (mixture experiment with glyphosate)
	0.44 (0.05-0.92) (mixture experiment with dimethoate)

3.3.2.1 Mixture exposure – *Porcellionides pruinosus*

Data from the combination of glyphosate and spirodiclofen was fitted to the CA model, which resulted as the best descriptive model (Table 3.4) But when the same data were fitted to the IA model, a dose level dependent deviation from the reference conceptual model was found with the negative parameter *a* indicating that synergism occurs at low dose levels of both pesticides and antagonism occurs at higher doses of both pesticides. The parameter *b* was higher than 1, thus indicating that the change between synergism and antagonism takes place at lower doses than the AC_{50} of the pesticides (Table 3.4).

For the dimethoate and glyphosate exposure experiment, after fitting the data to the CA model an antagonistic deviation from the reference model was observed by the positive parameter *a*, meaning that the predicted escape response of the animals was lower than the expected considering their individual response. After fitting the data to the IA model, the most parsimonious result was the reference model and no deviations were observed (Table 3.4).

Data from the combined experiment of dimethoate and spirodiclofen fitted to both reference models, translated into an antagonistic deviation. After fitting the data to the CA model, antagonism was observed (Table 3.4). After using the IA model, an antagonistic pattern was also observed as the parameter *a* was also higher than zero (Table 3.4).

Table 3.4: Parameter estimates and tests of fit of the reference models using the MIXTOX model applied to the behavioural avoidance response of *Porcellionides pruinosus* exposed for 48h to three pesticide mixtures in LUFA 2.2 soil.

		Concentration Addition					Independent Action				
Mixture		r^2	p (χ^2)	SS	a	b	r^2	p (χ^2)	SS	a	b
Glyphosate and Spirodiclofen	Reference	0.19	***	100.6	-	-	-	-	-	-	-
	S/A	-	-	-	-	-	-	-	-	-	-
	DR	-	-	-	-	-	-	-	-	-	-
	DL	-	-	-	-	-	0.24	*	93.1	-19.8	115.9
Dimethoate and Glyphosate	Reference	-	-	-	-	-	0.23	**	61.3	-	-
	S/A	0.27	*	58.3	4.5	-	-	-	-	-	-
	DR	-	-	-	-	-	-	-	-	-	-
	DL	-	-	-	-	-	-	-	-	-	-
Dimethoate and Spirodiclofen	Reference	-	-	-	-	-	-	-	-	-	-
	S/A	0.46	*	22.1	7.1	-	0.48	*	21.2	5.2	-
	DR	-	-	-	-	-	-	-	-	-	-
	DL	-	-	-	-	-	-	-	-	-	-

r^2 is the coefficient of determination; p (χ^2) indicates the outcome of the likelihood ratio test (significance levels: * <0.05; **<0.01; ***<0.001); SS is residuals sum of squares; *a* and *b* are the parameters of the deviations; S/A is synergism/antagonism, DR is dose-ratio deviations and DL is dose-level deviation from the reference models.

3.3.2.2 Mixture exposure – *Folsomia candida*

After fitting the data from the combination of glyphosate and spirodiclofen to the CA model a deviation for antagonism was found (Table 3.5) The same antagonistic deviation was observed when this data was fitted to the IA conceptual model since once again the parameter *a* was higher than zero (Table 3.5).

For the dimethoate and glyphosate exposure experiment, after fitting the data to the CA model an antagonistic deviation from the reference model was observed, with a positive parameter *a* meaning that the predicted toxicity of the mixture was lower than the expected considering the individual exposure effects. After fitting the data to the IA model, the same result was obtained, i.e. a deviation for antagonism (Table 3.5).

When data of the binary experiment of dimethoate and spirodiclofen was fitted to the CA model, the most parsimonious result was the reference model. After fitting our data to the IA model, the most parsimonious model was also the reference model itself and no deviations were obtained, since subsequent addition of extra parameters did not improve the fitness of the model (Table 3.5).

Table 3.5: Parameter estimates and tests of fit of the reference models using the MIXTOX model applied to the reproductive output of *Folsomia candida* exposed for 28 d to three pesticide mixtures in LUFA 2.2 soil.

		Concentration addition					Independent Action				
Mixture		r^2	p (χ^2)	SS	a	b	r^2	p (χ^2)	SS	a	b
Glyphosate and Spirodiclofen	Reference	-	-	-	-	-	-	-	-	-	-
	S/A	0.68	***	395501.1	3.9	-	0.67	**	399954.6	3.9	-
	DR	-	-	-	-	-	-	-	-	-	-
	DL	-	-	-	-	-	-	-	-	-	-
Dimethoate and Glyphosate	Reference	-	-	-	-	-	-	-	-	-	-
	S/A	0.61	*	477742.1	2.3	-	0.64	*	438094.4	2.9	-
	DR	-	-	-	-	-	-	-	-	-	-
	DL	-	-	-	-	-	-	-	-	-	-
Dimethoate and Spirodiclofen	Reference	0.77	***	372975.3	-	-	0.76	***	400299.9	-	-
	S/A	-	-	-	-	-	-	-	-	-	-
	DR	-	-	-	-	-	-	-	-	-	-
	DL	-	-	-	-	-	-	-	-	-	-

r^2 is the coefficient of determination; SS is the residuals sum of squares; p (χ^2) indicates the outcome of the likelihood ratio test (significance levels: * <0.05; **<0.01; ***<0.001); *a* and *b* are the parameters of the deviations; CA is concentration additions; IA is independent actions; S/A is synergism/antagonism, DR is dose-ratio deviations and DL is dose-level deviation from the reference models.

3.4 Discussion

3.4.1 Single exposure toxicity – *Porcellionides pruinosus*

The AC_{50} calculated from the single exposure procedure and the single exposures in the combined experiments for the three pesticides were in the same range of values for the three pesticides tested (Table 3.2). In fact, in only two combinations (glyphosate with dimethoate and spirodiclofen with dimethoate) the values were two-fold the ones calculated in the single exposure procedure.

Single exposure to the herbicide glyphosate caused a clear avoidance response in the terrestrial isopod *Porcellionides pruinosus*. In a laboratory study where the direct mortality of the isopod *Philoscia muscurom* exposed to glyphosate application rates of 6 L/ha (≈ 2.1 Kg Ai/ha) evident effects on survival rate were observed (Eijsackers, 1985) and in a subsequent study, a decrease in the consumption rate of leaf litter treated with the same amount of herbicide was also observed (Eijsackers, 1991). In a laboratory study with the earthworm *Aporrectodea caliginosa* treated at levels equivalent to application rates of 0.7–2.8 Kg glyphosate/ha, a decrease in growth rates and early mortality of the earthworms were observed (Springett and Gray 1992). In the present study the concentration that caused an avoidance response in *Porcellionides pruinosus* was almost 17 times higher than the predicted environmental concentration (PEC) in the top 10 cm soil (3060 g AI/ha ≈ 2.5 mg/Kg dry soil) if the commercial formulation is applied at labeled doses.

Previous studies with the same isopod species and dimethoate obtained very similar AC_{50} values as these reported here, in individual (39.43 mg/Kg) and collective (28.67 mg/kg) avoidance tests (Loureiro et al., 2005). Another study dealing with the influence of dimethoate to the isopod *Porcellio scaber* (Engenheiro et al., 2005) observed a reduction in 50% of the survival rate of this organism after 10 days of exposure to 20 mg/Kg. According to the same study dimethoate clearly influenced the locomotor behaviour of *P. scaber*, related with a significant decrease in acetylcholinesterase (AChE) activity, since this OP insecticide is a well known inhibitor of this nervous system enzyme (Bayley, 1995). The results obtained in the present study seem to indicate that this insecticide exerts a clear dose-related behavioural pattern in *P. pruinosus*. However it should be noted that

the PEC value (400 g AI/ha ≈ 0.3 mg/Kg dry soil) is much lower than the concentrations causing an avoidance response in this species. In the two highest dimethoate concentrations tested, more than 80% of the animals were found in the control side of the test boxes. This situation represents a loss in the "habitat function" of the soil (Hund-Rinke et al., 2003) and should be taken into account in evaluating the detrimental effects in isopods population after dimethoate application in agricultural fields. The effects of dimethoate on the species *P. scaber* have also been assessed in terms of mortality (Løkke and Van Gestel, 1998) and growth pattern (Fischer et al., 1997), and the values obtained for these two parameters, 75 mg/Kg and 17.5 mg/Kg respectively, can be considered in the same range of the AC_{50} values reported here.

Our results indicated that spirodiclofen had an impact in the avoidance behaviour of *P. pruinosus*, being the calculated AC_{50} much lower (0.91 mg/Kg) than the values obtained for the other two pesticides tested. Nevertheless it should be pointed out that the AC_{50} is more than 10 times higher than the predicted environmental concentration in the top 10 cm soil (PEC) if spirodiclofen is applied at recommended field rates (96 g AI/ha ≈ 0.06 mg/Kg dry soil). At 0.27 mg spirodiclofen/Kg dry soil, which is below spirodiclofen response threshold, more animals were found in the contaminated side of the box. Possibly this could be related with the attraction that some substance of the formulation exerted on isopods (Olla et al., 1980; Zimmer et al., 1996). This finding (non-avoidance of contaminated soil) has been described in other experiments with non-narcotic chemicals (Yeardley et al., 1996; Odendaal and Reinecke, 1999) and with narcotic chemicals (Heupel et al., 2002; Landrum et al., 2003) in different test species.

After an application rate of the commercial formulation Envidor® in an open field, spirodiclofen was considered very toxic to *Tetranychus urticae* and moderately toxic to *Tarsodemus pallidus* (Raudomis, 2006). All these results seem to confirm the toxicity of this insecticide, thus attention should be paid in analyzing possible damages in non-target organisms like the macro decomposers terrestrial isopods, following the application of this product to agricultural crops.

3.4.2 Single exposure toxicity of *Folsomia candida*

The EC_{50} value calculated for the insecticide dimethoate (in the single experiments performed simultaneously to the binary combinations) was six times lower than the value obtained in the single exposure procedure, although the value for glyphosate can be considered in the same range of values obtained in the single exposure procedure (Table 3.3). The same can be said about the insecticide spirodiclofen, since the EC_{50} values obtained were in the same range of concentration.

The effects of the application of the herbicide glyphosate to collembolan populations have been assessed in field plot experiments. One study showed that the application of this herbicide favoured the appearance of epigeic and hemiedaphic species due to the preservation of weed cover given by glyphosate (Renaud et al, 2004). A previous study has also demonstrated the indirect effect of this herbicide on the enhancement of biological activity in soils, since the supply of organic matter (due to the killing of the cover crop) had a positive influence on the feeding activity of soil microbial communities (Reinecke et al., 2002). However, other studies dealing with different herbicide and collembolan species showed contrasting results. A field and laboratory test observed that atrazine reduced the abundance of *Entomobrya musatica* (AI-Assiuty and Khalil, 1998) at doses near the recommended application rate of this herbicide. Another study also detected effects on the reproductive capacity of collembolan at herbicides concentrations below the mortality threshold (Chernova et al., 1995). The impairment in the reproductive output of *Folsomia candida* detected in the present work clearly indicates that the application of this herbicide does have an impact in both the survival and the reproductive capacity of this collembolan species.

The effects of the insecticide dimethoate on the reproduction of the collembolan *Folsomia candida* have been assessed previously. Krogh (1995) have found that the application of dimethoate near the recommended dose had an impact in the reproductive output (EC_{50} of 0.5 (0.4-0.6) mg/Kg) and survival rate of adults (LC_{50} of 0.6 (0.6-0.6) mg/Kg). Another work dealing with the same insecticide applied to three different soil types (Martikainen, 1996) derived EC_{50} values between 3.8-6.3 mg/Kg, which were attributed to the high organic content of the soil used, responsible for the reduction of dimethoate toxicity. More recent studies have attested dimethoate toxicity and its impact

on the reproductive capacity of *F. candida* (Sørensen and Holmstrup, 2005). The capacity to predict the effect of pollutants in dynamic natural population can be regarded as the main objective of ecotoxicological studies (Moe et al., 2001), so it seems pertinent to address the question if dimethoate could in long term exposure periods cause a disruption in the population dynamics of this collembolan species.

Since the DT_{90} of spirodiclofen and its soil metabolites is less than 100 days no testing with soil non-target macro-organisms was triggered for regulatory purposes, however this pesticide was considered of low risk to earthworms and to the collembolan species *Folsomia candida* when applied at recommended doses in agricultural fields (Candolfi et al, 2001). The EC_{50} calculated for spirodiclofen was 0.65 (0.37-0.96) mg/Kg, which represents 10 fold of the PEC if Envidor® is applied at the labelled recommended dose. It should be noted, however, that the field soil dissipation of this product is considered to be very fast (DT_{50} between 0.5–5.5 days), thus in agricultural systems the period in which this product is available should be taken into account when inferring the effects this pesticide may have to edaphic organisms.

3.4.3 Mixture exposure toxicity

3.4.3.1 Mixture exposure toxicity – *Porcellionides pruinosus*

Of the three binary combinations tested only when the two insecticides, dimethoate and spirodiclofen, were applied together the same deviation (antagonism) was obtained from the two reference models. In the other two binary combinations the predictions were not the same, although they were not discordant, in view of the fact that in both cases CA or IA did not predict a deviation from the conceptual models.

The exposure to the combination of glyphosate and spirodiclofen followed the conceptual model of CA, hence their combined effects produce a predictable response based on the measured effects of single chemicals (Faust et al., 1993). This simple additive effect was also registered in an experiment performed with mixtures of an herbicide and an insecticide (atrazine and lindane) in a freshwater microcosm (Van den Brink et al., 2009),

where it was concluded that the toxic effects of the binary mixture could be explained by the effects of the individual chemicals alone.

After fitting the IA model to the same data, a dose level deviation was found, indicating synergism at low doses of the two pesticides and antagonism with increasing doses (higher than the respective AC_{50}) of the two pesticides. This means that the simple additive effect predicted by CA was not corroborated by the IA model; instead a deviation from this reference model was detected, hence the probability of response (avoiding the contaminated soil) to the two chemicals was not independent. Synergism found at low doses of the two pesticides is an indication of higher escape response than expected from the individual action of both chemicals, and thus may represent an hazard for the communities exposed to the two pesticides.

Dimethoate and glyphosate behavioural data when fitted in the CA model translated into antagonism, predicting a smaller effect of the two pesticides than expected. When this data was fitted in the IA model, it followed the conceptual model attesting the *a priori* knowledge that these pesticides have dissimilar modes of action. A previous study performed with the isopod *Porcellionides pruinosus* with binary mixtures of dimethoate and the triazine herbicide atrazine predicted antagonism at low doses of both pesticides and a switch to synergism at high doses of both pesticides after fitting data to the IA model (Loureiro et al., 2009). This deviation is in agreement with the antagonistic effect found in the present work, although predicted by the other reference model used in mixture toxicity assessment.

Regarding the same mixture data, no deviation from the conceptual model of IA was found in the same study when this reference model was applied. Previous studies dealing with binary mixtures of atrazine and OP insecticides have detected a synergistic effect of the herbicide to the toxicity of chlorpyrifos and diazinon to the aquatic organisms *Chironomus tentans* and *Hyalella azteca* (Jin-Clark et al., 2002; Anderson and Lydy, 2002). Similar studies performed with *Chironomus tentans* midges found that the dimethoate toxicity is enhanced due to the oxidative activation process brought by the action of the herbicide atrazine on the cytochrome P450 monooxygenase (Cyt-P450) enzymatic complex (Anderson and Zhu, 2004). This molecular activation of dimethoate due to the action of the herbicide atrazine causes a cascade of reactions that culminate in

the increase inhibition of AChE activity, resulting in a higher toxicity of the two pesticides to the chironomid midges. In the present work, apparently, this molecular activation of the insecticide dimethoate did not occur, and this can be explained by the different molecular mechanism of action of the herbicide glyphosate in comparison with atrazine (although known only for plants). The same synergistic effect was found in a more recent work with the same test-species, where the action of two chloroacetanilide herbicides (alachlor and metolachlor) on chlorpyrifos was observed (Jin-Clark et al., 2008). These herbicides reduced the activity of the detoxifying enzyme glutathione-S-transferase (GST) which led to the interruption of metabolic detoxification of chlorpyrifos, thus increasing the quantity of this insecticide inside the organism and consequently increasing the peril of intoxication.

The effect of dimethoate and spirodiclofen on *P. pruinosus* avoidance behaviour showed an identical deviation from the two reference models, since in both models antagonism was predicted. The combined effect of dimethoate and the organochlorine insecticide lindane was also assessed for this isopod species, and deviations from the two reference models were also observed (Loureiro et al., 2009). In the cited work, it was observed that after fitting the avoidance data of dimethoate and lindane to CA model a dose ratio dependency did occur, with higher toxicity than expected found when lindane was the dominant component in the mixture; but when the same data was fitted into the IA conceptual model a dose level deviation was observed, with antagonism at low doses and synergism with increasing doses (higher than the AC_{50}) of both pesticides. The prediction obtained when testing the IA model was in agreement with the results found in the present work, given that antagonism was detected at smaller doses of both pesticides and the synergism observed was registered only at higher levels of both pesticides, thus its biologically relevancy can be questioned since at higher doses the animal could not survive the exposure concentrations.

3.4.3.2 Mixture exposure toxicity - *Folsomia candida*

There was a decrease in the number of juveniles with increasing toxicity of the binary combinations tested, but the degree in which the number of juveniles diminished

was not as high as expected by the results obtained in the single exposure procedures run simultaneously. Individual concentrations of glyphosate and spirodiclofen originated a strong and abrupt decrease in the number of juveniles produced (reduction in 90% of the juveniles produced in doses higher but near the EC_{50} in comparison to the control), but when the same concentrations were tested in the binary mixtures (sum of TU between 1 and 2) the reduction in the number of juveniles compared to the control was between 70% and 60% in the mixture of glyphosate and spirodiclofen. The same happened in the binary mixtures of dimethoate and glyphosate, since when both toxicants were individually present at concentrations higher but near the respective EC_{50} the decrease in the number of juveniles was between 85% and 90% of the offspring observed in control, whereas in the binary combinations with correspondent concentrations the decrease was between 85% and 50% in comparison to the control. As a consequence of this decrease that was not as steeper as the expected, and regarding the observed individual concentrations, antagonism was predicted for these two binary mixtures.

Glyphosate enhanced both glutathione-s-transferase (GST) and superoxide dismutase (SOD) enzymatic activity in the aquatic oligochaete *Lumbricus variegatus* submitted to a commercial formulation of this herbicide (Jara et al., 2009). The two antioxidant enzymes that are related with detoxifying xenobiotics and cellular stress were both activated at non-toxic levels of this herbicide. In a study with a selected strain of the two spotted spider mite *Tetranychus urticae*, it was observed that increased GST and cyt-P450 activity led to a spirodiclofen resistance factor of 13 (Rauch and Nauen, 2003). This could conduct us to hypothesize the possible biochemical interactions between the two pesticides, since the enhanced activity caused by glyphosate in the cited enzymatic complexes could lead to an increasing resistance to spirodiclofen toxic effects in the target organism. A work testing the efficacy of IA in describing several dissimilar binary combinations demonstrated that IA was the most parsimonious model to describe 6 out of the 10 combinations tested, but antagonism was observed in the binary combination of chlorpyrifos and the fungicide inhibitor of cyt-P450 prochloraz (Martin et al., 2009). This was stated to be a result of the inhibition of the cyt-P450 complex by the fungicide, which could lead to an inactivation of chlorpyrifos into its more toxic form. Again, there was a correlation between biochemical changes in enzymes involved in cellular stress caused by one chemical and on subsequent magnitude of insecticide toxicity to organisms.

A previous study dealing with the combined effect of the herbicide atrazine with five different OP insecticides and one organochlorine insecticide to aquatic midge larvae (*C. tentans*) was evaluated using the IA model (Pape-Linstrom and Lydy, 1997). Their results predicted synergism (effects greater than additive) in four of the OP insecticides (chlorpyrifos, malathion, methyl-parathion, and trichlorfon) and antagonism when the herbicide was mixed with mevinophos. In that work a consistent and significant enhancement in toxicity of the majority OP insecticides due to the action of atrazine was observed, and several hypotheses were raised from an increasing cellular permeability of the midge cuticle caused by atrazine to the activation of cyt-P450 by the herbicide. An acute experiment (filter-paper test) made with the earthworm *Eisenia fetida* (Lydy and Linck, 2003) found that when chlorpyrifos was mixed with atrazine and cyanazine these herbicides increased the toxicity of chlorpyrifos by a factor of 7.9 and 2.2 respectively. Still, when chlorpyrifos was mixed with symazine this herbicide did not affect the insecticide toxicity, probably as a result of a non-activation of the insecticide. In an experiment carried out in the field, mite and ant populations were reduced after application of herbicides (nicosulfuron and atrazine) mixed with chlorpyrifos (Pereira et al., 2005). However, an opposite conclusion was derived from exposure of the larvae of *Cacopsylla pyri* to atrazine where this herbicide led to a decrease in mortality following an exposure to the insecticide *Bti* (Boyer et al., 2006).

From the studies described above one must admit that further knowledge of the pesticides mode of toxic action should be established to make more assertive assumptions about the mechanisms involved in the combined toxicity of herbicides and insecticides to soil organisms. In the lack of systematic understanding of the mechanisms underlying the toxic effects on the specific test-organism, only general considerations could be made about mixture toxicity interaction inside the target organisms.

The mixture of dimethoate and spirodiclofen followed the two conceptual models when data was fitted to CA and IA. This means that the effects obtained when these two insecticides were applied jointly could be expressed as simple dilution of each other (after CA conceptual model), implying that one insecticide could be replaced by a dilution of the other without alterations in the resultant toxicity (Berenbaum, 1989). The same result was obtained in previous studies dealing with the effects of insecticides of similar action

(AChE inhibitors dimethoate and pirimicarb) on *Daphnia magna*, where the binary mixture followed CA, but IA could also explain the data reasonably well (Syberg et al., 2008).

3.4.4 Comparison between avoidance and reproduction tests

Although the two tests evaluated different test species, different parameters (avoidance behaviour and reproduction output) and different exposure periods a comparison between the outcome of the two test should be established. Looking at the AC_{50} values of glyphosate and dimethoate the median values were more than 100-times higher than the EC_{50} calculated for the collembolan reproduction. Only in the case of spirodiclofen the values can be considered in the same range interval. The discrepancy between the AC_{50} and EC_{50} values could be explained by the different sensitivity to PPPs application, since the isopods move mainly in the soil surface and have clean (uncontaminated) soil to where they can escape and collembola are buried in contaminated soil during the test period. In addition, avoidance test often gave higher ECx values than reproduction chronic tests (Amorim et al., 2005), which could be related with the short period used in avoidance test (48 h) in comparison to the 28 days in reproduction tests. Thus avoidance behaviour tests can be used as initial screening test in soil contamination assessment and not as a substitute of reproduction tests (Loureiro et al., 2005).

3.5 Conclusions

From an overall observation of the results obtained from fitting the data of avoidance behaviour and reproductive output to both models several assumptions can be made regarding the ability of the two conceptual models in predicting mixture toxicity. Data from the mixtures followed the reference models in 4 out of the 12 combinations tested (see Table 3 and Table 4). In 7 out of the 12 mixtures performed the two reference models predicted antagonism. In only one combination (glyphosate and spirodiclofen applied to *P. pruinosus*) synergism was observed. There was a general agreement between the two reference models in predicting mixture toxicity, with the exception of two mixtures

tested. As a result of this, both models could and should be used to address mixture toxicity problems, since the information gathered from fitting data to both models was complementary and never contradictory, in order to understand what happens when plant protection products are applied together.

References

Al-Assiuty, A.I.M., Khalil, M.A., 1998. Effects of the herbicide atrazine on *Entomobrya musatica* (Collembola) in field and laboratory experiments. App. Soil Ecol. 4, 139-146.

Aldaya, M.M., Lors, C., Salmon, S., Ponge, J.F., 2006. Avoidance bio-assays may help to test the ecological significance of soil pollution. Environ. Pollut. 140, 173-180.

Amorim, M.J.B., Römbke, J., Soares, A.M.V.M., 2005. Avoidance behaviour of *Enchytraeus albidus*: Effects of benomyl, carbendazim, phenmedipham and different soil types. Chemosphere 59, 501-510.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008. Avoidance test with *Enchytraeus albidus* (Enchytraeidae): Effects of different exposure time and soil properties. Environ. Pollut. 155 (1), 112-116.

Anderson, T.D., Lydy, M.J., 2002. Increased toxicity to invertebrates associated with a mixture of atrazine and organophosphate insecticides. Environ. Toxicol. Chem. 21, 1507–1514.

Anderson, T.D., Zhu, K. Y., 2004. Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera: Chironomidae). Pestic. Biochem. Physiol. 80, 54–64.

Baas, J., van Houte, B.P.P., van Gestel, C.A.M., Kooijman, S.A.L.M., 2007. Modelling the effects of binary mixtures on survival in time. Environ. Toxicol. Chem. 26 (6), 1320-1327.

Bayley, M., 1995. Prolonged effects of the insecticide dimethoate on locomotor behavior in the woodlouse *Porcellio scaber* Latr. (Isopoda). Ecotoxicology 4, 79-90.

Berenbaum, M.C., 1989. What is synergy. Pharmacol. Rev. 41(2), 93-141.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 585–615.

Boyer, S., Sérandour, J., Lempérière, G., Raveton, M., Ravanel, P., 2006. Do herbicide treatments reduce the sensitivity of mosquito larvae to insecticides? Chemosphere 65, 721–724.

Candolfi, M. P., Barrett, K. L., Campbell, P. J., Forster, R., Grandy, N., Huet, M. C., Lewis, G., Oomen P. A., Schmuck, R., and Vogt, H., 2001. Guidance document on regulatory testing and risk assessment procedures for plant protection products with non-target arthropods. Proceedings of a Workshop held in Wageningen from 21 to 23 March 2000.

Chernova, N.M., Balabina, I.P., Ponomareva, O.N., 1995. Changes in population growth of springtail (Collembola) under the influence of herbicides. Polske. Pismo. Entomol. 64, 91-98.

Crouau, Y., Chenon, P., Gisclard, C. 1999. The use of *Folsomia candida* (Collembola, Isotomidae) for the bioassay of xenobiotic substances and soil pollutants. Appl. Soil Ecol. 12(2), 103-111.

Eaton, R.J., 2006. Collembola population levels 7 years after installation of the North Carolina Long Term Soil Productivity Study. Pedobiologia 50, 301-306.

Eijsackers, H., 1985. Effects of glyphosate in soil fauna, in: Grossbard, E and Atkinson, D. (Eds.), The herbicide glyphosate,, Butterworths, London, pp 151-157.

Eijsackers H. 1991. Litter fragmentation by isopods as affected by herbicide application. Neth. J. Zool. 41(4), 277-303.

Engenheiro, E.L., Hankard, P.,K., Sousa, J.P., Lemos, M.F.L., Weeks, J.M., Soares, A.M.V.M., 2005. Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environ. Toxicol. Chem. 24 (3), 603-609.

Faust, M., Altenburger, R., Boedecker, W. and Grimme, L.H., 1993. Additive effects of herbicide combinations on aquatic non-target organisms. Sci. Total Environ. (Suppl.) 941-952.

Ferreira, A.L.G., Loureiro, S., and Soares, A.M.V.M., 2008. Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on *Daphnia magna*. Aquat. Toxicol. 89, 28-39.

Fischer, E., Farkas, S., Hornung, E., Past, T., 1997. Sublethal effects of an organophosphorus insecticide, dimethoate, on the isopod Porcellio scaber Latr. Comp. Biochem. Physiol. 116 C (2), 161-166.

Gomez-Eyles, J.L., Svendsen, C., Lister, L., Martin, H., Hodson, M.E., Spurgeon, D.J., 2009. Measuring and modelling mixture toxicity of imidacloprid and thiacloprid on *Caenorhabditis elegans* and *Eisenia fetida*. Ecotox. Env,. Saf. 72 (1), 71-79.

Greenslade P. & Vaughan G.T., 2003. A comparison of Collembola species for toxicity testing of Australian soils. Pedobiologia 47, 171-179.

Heupel, K., 2002. Avoidance response of different collembolan species to Betanal. Eur. J. Soil Biol., 38, 273-276.

Hund-Rinke, K., Achazi, R., Römbke, J., Warnecke, D., 2003. Avoidance Test with *Eisenia fetida* as indicator for the habitat function of soils: results of a laboratory comparison test. J. Soils Sed. 3 (1), 1-6.

Hewlett, P.S., Plackett, R.L., 1959. A unified theory for quantal responses to mixtures of drugs: non-interactive action. Biometrics 15, 591-609.

ISO, 1999. Soil quality – inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants. ISO/DIS 11267. International Standardization Organization, Geneva, Switzerland.

ISO, 2008. Soil quality – avoidance test for determining the quality of soils and effects of chemicals on behaviour – Part 1: Test with earthworms (*Eisenia feti*da and *Eisenia andrei*). ISO/DIS 17512-1. International Standardization Organization, Geneva, Switzerland.

ISO, 2010. Draft:. Soil quality – avoidance test for determining the quality of soils and effects of chemicals on behaviour – Part 2: Test with collembolans (*Folsomia candida*). ISO/DIS 17512-2. International Standardization Organization, Geneva, Switzerland.

Jänsch S., Amorim M. & Römbke J. (2005). Identification of the ecological requirements of important terrestrial ecotoxicological species. Environ. Rev. 13, 51-83.

Jara, V.C., Klingelmann, E., Wiegand, C., 2009. Bioaccumulation of glyphosate and its formulation Roundup Ultra in *Lumbriculus variegatus* and its effects on biotransformation and antioxidant enzymes. Environ. Pollut. 157, 57-63.

Jin-Clark, Y., Lydy, M.J., Zhu, K.Y., 2002. Effects of atrazine and cyanazine on chlorpyrifos toxicity in *Chironomus tentans* (Diptera: Chironomidae), Environ. Toxicol. Chem. 21, 598–603.

Jin-Clark, Y., Anderson, T.D., Zhu, K.Y., 2008. Effect of alachlor and metolachlor on toxicity of chlorpyrifos and major detoxification enzymes in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). Arch. Environ. Contam. Toxicol. 54, 645–652.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.M., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Env. Toxicol. Chem. 24, 2701-2713.

Junghans M, Backhaus T, Faust M, Scholze M, Grimme LH. 2006. Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. Aquat Toxicol., 76, 93–110.

Kautz, G., Topp, W., 2000. Acquisition of microbial communities and enhanced availability of soil nutrients by the isopod Porcellio scaber (Latr.) (Isopoda: Oniscidea). Biol. Fertil. Soils, 31:102–107.

Krogh, P.K., 1995. Does a heterogeneous distribution of food or pesticide affect the outcome of toxicity test with collembola? Ecotoxicol. Environ. Saf. 30, 158-163.

Landrum, P.F., Lotufo, G.R., Gossiaux, D.C., Gedeon, M.L., Lee, J.H., 2003. Bioaccumulation and critical body residue of PAHs in the amphipod, Diporeia spp.: additional evidence to support toxicity additivity for PAH mixtures. Chemosphere 51, 481-489.

Larsen, J.C., Binderup, M.-L., Dalgaard, M., Dragsted, L.O., Hossaini, A., Ladefoged, O., Lam, .R., Madsen, C., Meyer, O., Rasmussen, E.S., Reffstrup, T.K., Søborg, I., 1346 Vinggaard, A.M., Østergård, G., 2003. Combined actions and interactions of 1347 chemicals in mixtures. The toxicological effects of exposure to

mixtures of 1348 industrial and environmental chemicals. In: Larsen, J.C. (Ed.). Danish Veterinary 1349 and Food Administration. FødevareRapport. p. 12.

Loewe, S., Muischnek, H., 1926. Effect of combinations: mathematical basis of problem. N-S. Arch. Ex. Path. Ph. 114, 313–326.

Løkke, H., Van Gestel, C.A.M., 1998. Handbook of Soil Invertebrate Toxicity Tests. John Wiley & Sons Publisher, Chichester.

Loureiro, S., Amorim, M.J.B., Campos, B., Rodrigues, S.M.G., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157 (2), 625-632.

Loureiro, S., Soares, A.M.V.M., Nogueira, A.J.A., 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ. Pollut. 138, 121-131.

Lydy, M.J., Linck, S.L., 2003. Assessing the impact of triazine herbicides on organophosphate insecticide toxicity to the earthworm *Eisenia fetida*. Arch. Environ. Contam. Toxicol. 45, 343-349.

Martikainen, E., 1996. Toxicity of dimethoate to some soil animal species in different soil types. Ecotoxicol. Environ. Saf. 33 (2), 128–136.

Martin, H.L., Svendsen, C., Lister, L.J., Gomez-Eyles, J.L., Spurgeon, D.J., 2009. Measurement and modeling of the toxicity of binary mixtures in the nematode *Caenorhabidtis elegans* - a test for independent action. Environ. Toxicol. Chem. 28(1), 97-104.

Moe, S.J., Stenseth, N.C., Smith, R.H., 2001. Effects of a toxicant on population growth rates: sublethal and delayed responses in blowfly populations. Funct. Ecol. 15, 712–721

Munkegaard, M., Abbaspoor, M., Cedergreen, N., 2008. Organophosphorous insecticides as herbicide synergists on the green algae Pseudokirchneriella subcapitata and the aquatic plant *Lemna minor*. Ecotoxicology 17, 29-35.

Natal-da-Luz, T., Amorim, M.J.B., Römbke, J., Sousa, J.P., 2008. Avoidance tests with earthworms and springtails: Defining the minimum exposure time to observe a significant response. Ecotoxicol. Environ. Saf. 75, 545–551.

Nauen, R., 2005. Spirodiclofen:mode of action and resistance risk assessment in Tetranychid pest mites. J. Pestic. Sci. 30, 272–274.

Odendaal, J.P, Reinecke, A.J, 1999. The toxicity of sublethal lead concentrations for the woodlouse *Porcellio laevis* (Crustacea, Isopoda). Biol Fertil. Soils 29,146–151.

Olla, B.L., Pearson, W.H., Studholme, A.L., 1980. Applicability of behavioral measures in environmental assessment. Rapp. P-v Réun. Cons. Int. Explor. Mer. 179,162–173.

Owojori, O.J., Reinecke, A.J., 2009. Avoidance behaviour of two eco-physiologically different earthworms (*Eisenia fetida* and *Aporrectodea caliginos*a) in natural and artificial saline soils. Chemosphere 75, 279–283.

Pape-Lindstrom, P.A., Lydy, M.J., 1997. Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. Environ. Toxicol. Chem. 16, 2415–2420.

Pereira, J.L., Silva, A.A., Picanço, M.C., Barros, E.C., Jakelaitis, A., 2005. Effects of herbicide and insecticide interaction on soil entomofauna under maize crop. J. Environ. Sci. Health, Part B 40 (1), 45-54.

Petersen, H., 2002. General aspects of collembolan ecology at the turn of the millennium. Pedobiologia. 46, 246-260.

Rauch, N., Nauen, R., 2003. Spirodiclofen resistance risk assessment in *Tetranychus urticae* (Acari: Tetranychidae): a biochemical approach. Pest. Biochem. Physiol. 74, 91-101.

Raudomis, L., 2006. Comparative toxicity of spirodiclofen and lambdacihalotrin to *Tetranychus urticae*, *Tarsonemus pallidus* and predatory mite *Amblyseius andersoni* in a strawberry site under field conditions. Agron. Res. 4, 317-322.

Reddy, K.N., Rimando, A.M., Duke, S.O., Nandula, V.K., 2008. Aminomethylphosphonic acid accumulation in plant species treated with glyphosate. J. Agric. Food Chem. 56, 2125–2130.

Reinecke, A.J., Helling, B., Louw, K., Fourie, J., Reinecke, S.A., 2002. The impact of different herbicides

and cover crops on soil biological activity in vineyards in the Western Cape, South Africa. Pedobiologia. 46, 475–484.

Renaud, A., Poinsot-Balaguer, N., Cortet, J., Le Petil, J., 2004. Influence of four soil maintenance practices on Collembola communities in a Mediterranean vineyard. Pedobiologia 48: 623-630.

Röembke, J., 2008. Bioavailability in soil: The role of invertebrate behaviour. Dev. Soil. Sci. 12 (32), 245-260.

Skovlund, G., Damgaard, C., Bayley, M., Holmstrup, M., 2008. Does lipophicity of toxic compounds determine effects on drought tolerance of the soil collembolan *Folsomia candida*? Environ. Pollut. 144, 808-815.

Sørensen, T.S., Holmstrup, M., 2005. A comparative analysis of the toxicity of eight common soil contaminants and their effects on drought tolerance in the collembolan *Folsomia candida*. Ecotoxicol. Environ. Saf. 60, 132–139.

Springett, J.A., Gray, R.A.J., 1992. Effect of repeated low doses of biocides on the earthworm *Aporrectodea caliginosa* in laboratory culture. Soil Biol. Biochem. 24(12), 1739-1744.

Syberg, K., Elleby, A., Pedersen, H., Cedergreen, N., Forbes, V.E., 2008. Mixture toxicity of three toxicants with similar and dissimilar modes of action to *Daphnia magna*. Environ. Pollut. 69, 428-436.

Van den Brink, P.J., Crum, S.J.H., Gylstra, R., Bransen, F., Cuppen, J.G.M., Brock, T.C.M., 2009. Effects of a herbicide–insecticide mixture in freshwater microcosms: Risk assessment and ecological effect chain. Environ. Pollut. 157, 237–249.

Vieira, M.M., 2009. Vendas de produtos fitofarmacêuticos em Portugal em 2008. Direcção Geral de Agricultura e Desenvolvimento Rural. Ministério da Agricultura e do Desenvolvimento Rural, Portugal.

Wachendorff U, Nauen R, Schnorbach HJ, Rauch N and Elbert A., 2002. The biological profile of Spirodiclofen (Envidor®) – a new selective tetronic acid acaricide. Bayer Crop Sci J. 73 (55) 149–176.

Yeardley, R.B., Lazorchak, J.M., Gast, L.C., 1996. The potential of an earthworm avoidance test for evaluation of hazardous waste sites. . Environ. Toxicol. Chem 15, 1532-1537.

Zimmer, M., Kautz, G., Topp, W., 1996. Olfaction in terrestrial isopods (Crustacea: Oniscidea): Responses of *Porcellio scaber* to the odour of litter. Eur. J. Soil Biol. 32, 141-147.

Zimmer, M., 2002. Nutrition in terrestrial isopods (Isopoda:Oniscidea): an evolutionary– ecological approach. Biol. Rev. 77: 455–493.

4. THE JOINT TOXICITY OF THREE PLANT PROTECTION PRODUCTS TO *TRITICUM AESTIVUM* (L.) AND *BRASSICA RAPA* (L.)

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Abstract

Few studies have been conducted concerning the evaluation of mixtures of chemicals in the terrestrial environment. Although it is evident that organisms are submitted to more than just one stress condition in real exposure scenarios. The effects of binary combinations of three plant protection products (PPPs), the post-emergence herbicide glyphosate, the organophosphorous insecticide dimethoate and the acaricide spirodiclofen on the growth pattern of two species of plants (*Brassica rapa* and *Triticum aestivum*) were evaluated by the two reference models for mixture toxicity: concentration addition (CA) and independent action (IA). Results of single exposure to the three pesticides indicated a decrease in shoot length (SL) and fresh weight (FW) of the two plant species only at the highest concentrations tested of the three PPPs. In the combined experiments synergism was only found in the combination of glyphosate and dimethoate when the IA model was fitted to the SL and FW of *T. aestivum*. The results obtained in the present work seemed to corroborate that these PPPs have no detrimental effects when applied at recommended doses. In conclusion, and since synergism is the worrying condition in mixture toxicity assessment, it could be said that mixing and applying these PPPs at recommended doses did not lead to an increase in toxicity for these plant species, with the exception of the mixture of glyphosate and dimethoate applied to *T. aestivum*.

4.1 Introduction

Pollutants in soil systems rarely occur alone, thus the toxic effect of a single chemical to an organism is an improbable event, since organisms are exposed to many pollutants (Zwart and Posthuma, 2005). This is the case of agricultural fields where several plant protection products are applied at the same time (Santos et al., 2010). Since most studies in ecotoxicology are made by exposing a single test-species to a single chemical it seems important to evaluate the possible detrimental effects of the application of pesticide mixtures to non-target organisms (Yang, 1994).

Non-target plants can be affected by the application of pesticides to crops and by indirect drift of the products applied to destroy weeds and pests (Jong et al., 2008). In the present work two plant species were used to evaluate single and joint toxicity of Plant Protection Products (PPP): the monocotyledonous *Triticum aestivum* L. (wheat) and the dicotyledonous *Brassica rapa* L. (rapid life-cycle turnip) based on the available guidelines to study the effects of toxics in higher plants (GCPF, 2000; ISO, 1995; ISO, 2004), and also due to the prominent literature on the effects of several chemical on these two plant species (Kalsch et al., 2006; Song et al., 2007).

Two different concepts, concentration addition (CA), first developed by Loewe and Muishncek (1926) for chemicals with the same mode of action (MoA) and independent action (IA), later introduced by Bliss (1939), based on chemicals with different modes of action, are used in mixture toxicity assessment. Both models are non-interactive, since they assume that chemicals do no interact with each other in the mixture (Greco et al., 1992). But toxicokinetic and toxicodynamic interactions can occur between chemicals, and thus synergism (higher toxicity of the mixture than expected) or antagonism (smaller toxicity of the mixture than expected) can be observed when two or more chemicals are applied in a mixture (Andersen and Dennison, 2004).

Three PPPs were selected based on the market share that they have in Portugal and the rest of Europe (Vieira, 2009; Eurostat, 2007): the post-emergence herbicide glyphosate, the organophosphorous insecticide dimethoate, and the acaricide spirodiclofen. Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Steinrucken,

and Amrhein, 1980) deregulating the shikimate pathway which leads to general metabolic disruption in plants (Reddy et al, 2008). Dimethoate is one of the most commonly applied insecticides in agricultural fields, which mode of action in plants has been shown to be internal to the seeds through the inhibition of the synthesis or action of hydrolytic enzymes (Chopra and Nandra, 1969). Spirodiclofen is a selective, non-systemic acaricide from the novel class of tetronic acid derivatives, which interferes with lipid biosynthesis by inhibiting acetyl-Coa carboxylase (Nauen, 2005) although no effects in terrestrial plants were observed in previous laboratory testing to its commercial release (Wolf and Schnorbach, 2002).

The objectives of this work were firstly to determine the effects of three commonly used plant protection products on the growth pattern of *T. aestivum* and *B. rapa* and secondly to evaluate the combined effects of three binary mixtures of these pesticides. For this second aim, the MIXTOX tool will be used to model the CA and IA conceptual models and possible deviations.

4.2 Materials and Methods

4.2.1 Test organisms and test conditions

Seeds of *Brassica rapa* rapid cycle (Carolina Biological Supply Company) and *Triticum aestivum* (from a local supplier) were used in the experimental procedure. All experiments were conducted in a laboratory room, which was maintained at 20 ± 2°C (day/night). The relative humidity was between 40% and 60%. The room was maintained on a 16/8 h (day/night) cycle that provided a light intensity of about 7000 lux on the soil surface.

4.2.2 Test Chemicals and Test Soil

Three pesticides were used in the experimental procedure as commercial formulations: the post-emergence herbicide glyphosate (ROUNDUP® with 360 g AI/L, and

which contains glyphosate-isopropylammonium (45%), surfactant (16%) and water (42.5%)), the organophosphorous insecticide dimethoate (AGROR® with 400 g AI/L and which contains dimethoate (40 %), ciclohexanone (28.4%), nonylphenol ethoxylate (2.2%), petroleum naphta (26.1%) and calcium alkyl benzene sulphonate inpropil 2-ol (0.4%)) and the acaricide spirodiclofen (ENVIDOR® with 240 g AI/L which contains spirodiclofen (23.3%), ethoxylated polyarylphenol (1-22.5%) and glycerine (> 1%)). Pesticides were spiked in the soil LUFA 2.2 and immediately afterwards the seeds were sown at soil surface. The nominal concentrations used for glyphosate ranged from 1.7 to 173.4 mg/Kg dry; for dimethoate the nominal concentration used ranged from 0.2 and 40 mg/Kg dry soil; for spirodiclofen the nominal concentration used ranged from 0.04 and 2.7 mg/Kg dry soil.

All the tests were performed with LUFA 2.2 soil, commercialized by the German Institution LUFA Speyer. The properties of this soil include a pH=5.8, organic matter=3.9%, texture= 6% clay, 17% silt and 77% sand.

4.2.3 Experimental procedure

The continuous seed germination and seedling growth tests were performed following the procedures described in the ISO guideline 11269-2 (ISO, 1995) with some modifications. LUFA 2.2 soil, 450 g dry weight equivalent, was filled into plastic vessels. Each test vessel was 9 cm (top) or 6.5 cm (bottom) diameter and 6.5 cm height with a drain in the bottom filled with an 8 cm glass fiber wick (1 mm diameter). A plastic pot (9 cm diameter) filled with deionised water was placed underneath each test vessel in order to achieve automatic watering. Ten seeds were sown uniformly in each test vessel to a depth of about 0.5 cm. Seed germination was determined by visual seedling emergence and was recorded daily. After 50% of the seeds in control pots had germinated the test went on for more 14 days. The seedling shoots were then cut above the soil surface and the fresh biomass was immediately weighed. All experiments were carried out using five concentrations plus one control, using four replicates per treatment, in a total of 24 test vessels.

In the mixture experiments twenty-three binary combinations, based on the EC_{50} calculated in the individual tests, were made simultaneously with five single concentrations of each pesticide and one control, without replication, in a total of 34 test-boxes for each binary combination. The range of the sum of toxic units (TU) in the binary experiments went from 0.375 to a maximum of 2 (Fig. 4.1).

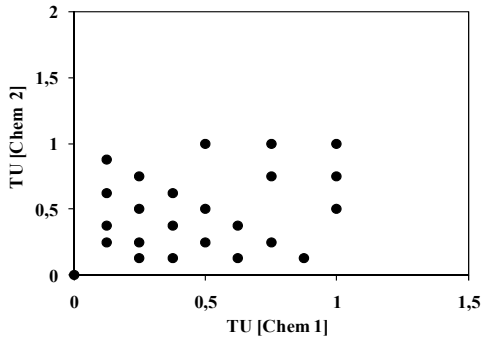

Fig. 4.1 – Experimental design of the binary combinations based on the toxic units (1 TU = EC_{50}) of glyphosate, dimethoate and spirodiclofen obtained in the single exposure tests. Data for [Chem 1] vs [Chem 2] refers to glyphosate vs dimethoate; glyphosate vs spirodiclofen; dimethoate vs spirodiclofen.

4.2.4 Statistical analyses

Differences in the fresh weight and shoot length of both plants between the control soil and the soil treatments were analysed using a one-way ANOVA, followed by *post-hoc* Dunnett´s test ($\alpha<0.05$). EC_{50} values were determined using appropriate non-linear models. For all the analysis mentioned above, STATISTICA 7.0 software was used (StatSoft Inc.).

The tool used to analyze and compare the data in the present study was the MIXTOX model (Jonker et al, 2005), which allow us to fit both reference models (CA and IA) to data, thus comparing the observed toxicity and the expected toxicity of the pesticides and also to calculate possible deviations from the two reference models. To test interactions between the two stressors, the MIXTOX model allows adding extra parameters (*a* and *b*) to confirm if deviations from the conceptual models are observed. The parameters are added in a stepwise manner, so parameter *a* is added first to see if

interactions have occurred in the mixture (Long et al., 2009). If the value of parameter *a* is higher than zero this means that a smaller effect than expected (antagonism) was observed; if the referred parameter *a* is smaller than zero than it expresses a higher effect than expected (synergism). If an improvement in the description of the data is obtained after adding parameter *a*, than this value is used along with parameter *b* in order to calculate further deviations from the reference models: dose ratio (DR) and dose level (DL) deviations. DR describes synergism/antagonism (if $a > 0$: antagonism except in mixtures where negative *b* values indicate synergism; if $a < 0$: synergism except in mixtures where negative *b* values indicate antagonism) according to the ratio of each chemical present in the mixture, and parameter *b* allows calculating if the antagonism/synergism is caused when one chemical is responsible for the toxicity of the mixture (see Table 4.1 for further information). DL describes synergism/antagonism depending on the level (concentration) of each stressor in the mixture. The value of parameter *a* allows to observe if synergism occurs at small doses and antagonism at high doses (parameter *a* smaller than zero) or if antagonism occurs at low doses and synergism at high doses (parameter *a* higher than zero). Parameter *b* will allow detecting if the change between the two deviations occurs at the EC_{50}, below the EC_{50} or above the EC_{50} level (see Table 4.1 for further information). The statistical improvement in explaining the data by adding parameters *a* and *b* is calculated using the chi-square test which will imply a decrease in the residuals of the sum of squares (SS) and an increase in the description potential of the variation of the data (r^2).

Table 4.1: Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation pattern from the reference models concentration addition (CA) and independent action (IA); adapted from Jonker et al. (2005).

Deviation Pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
synergism/antagonism (S/A)	**a>0:** antagonism **a<0:** synergism		
Dose-ratio dependent (DR)	**a>0:** antagonism except for those mixture ratios where negative b value indicate synergism	b_i**>0:** antagonism where the toxicity of the mixture is caused mainly by toxicant *i*	
	a<0: synergism except for those mixture ratios where positive b value indicate antagonism	b_i**<0:** synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	**a>0:** antagonism low dose level and synergism high dose level	b_{DL}**>1:** change at lower EC50 level	b_{DL}**>2:** change at lower EC50 level
		b_{DL}**=1:** change at EC50 level	b_{DL}**=2:** change at EC50 level
	a<0: synergism low dose level and antagonism high dose level	**0**<b_{DL}<**1:** change at higher EC50 level	**1**<b_{DL}<**2:** change at higher EC50 level
		b_{DL}<**1:** No change but the magnitude of S/A is DL dependent	b_{DL}<**1:** No change but the magnitude of S/A is effect level dependent

4.3 Results

4.3.1 Single Toxicity

Both endpoints, shoot length (SL) and fresh weigh (FW), evaluated during single exposure of *T. aestivum* and *B. rapa* to glyphosate showed that only at high doses significant differences were observed. The EC_{50} values for the single experiments and the single tests made simultaneously with the mixture experiments were very similar for SL and FW and close to the highest dosage of the herbicide tested (Fig. 4.2, Fig. 4.3, Table 4.2, and Table 4.3). The same occurred in the other two PPPs tested, dimethoate and spirodiclofen, since the EC_{50} calculated for SL and FW was close to the value of the highest concentration applied in the soil. Once more, the EC_{50} of the two endpoints evaluated can be considered in the same range of values (Fig. 4.2, Fig. 4.3, Table 4.2, and Table 4.3).

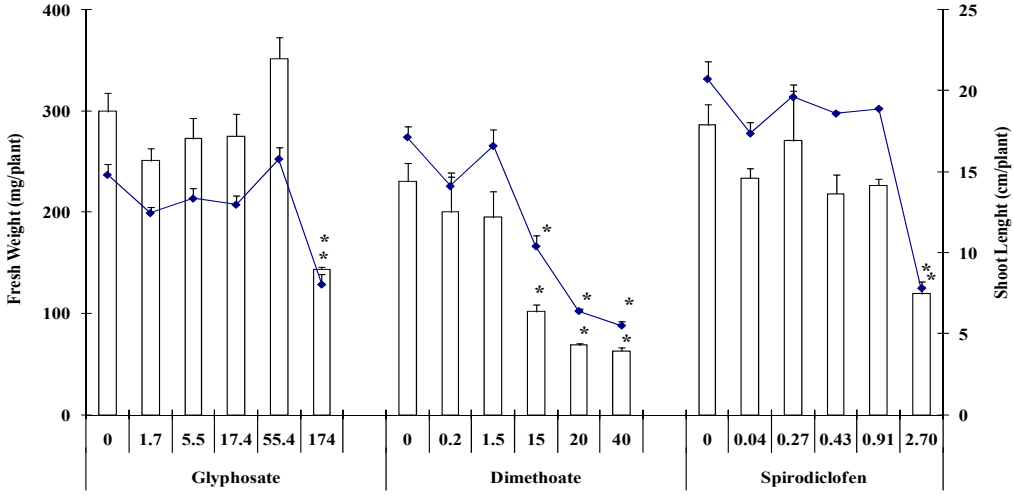

Fig. 4.2 – Fresh weight (columns) and shoot length (straight lines) of *Triticum aestivum* grown in LUFA 2.2 soil for 21 d, after application of glyphosate, dimethoate and spirodiclofen (mean net response with standard error bars). All units (nominal values) are in mg AI/Kg dry soil. (* indicates statistical differences (Dunnett´s method, $p<0.05$)).

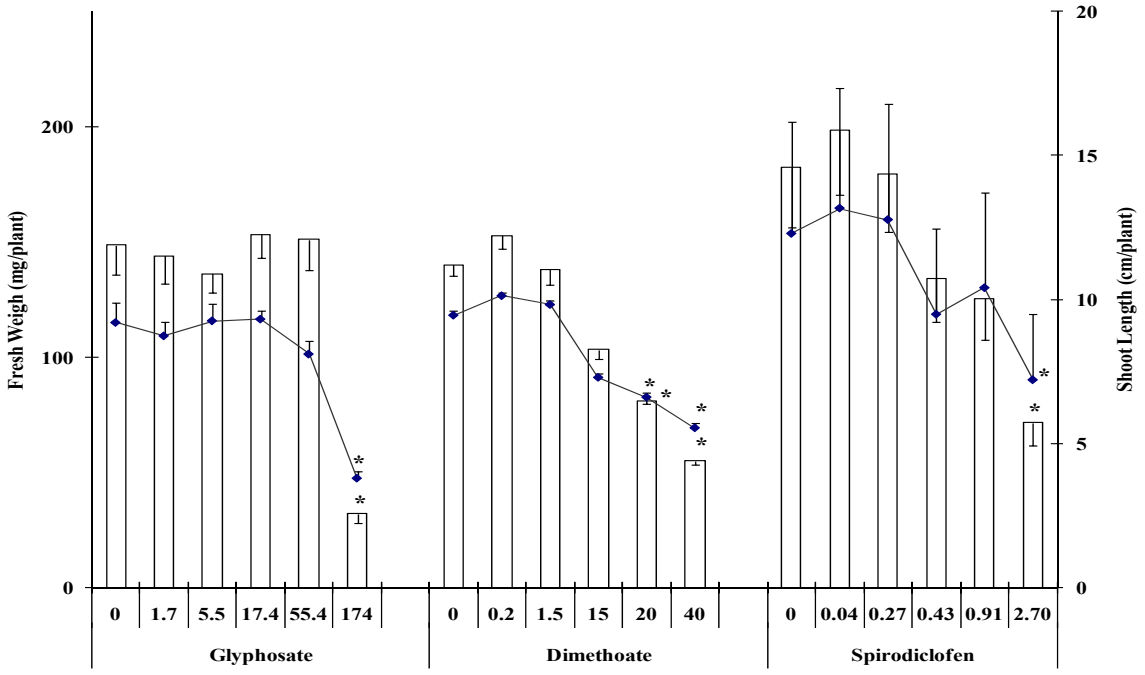

Fig. 4.3 – Fresh weight (columns) and shoot length (straight line) of *B. rapa* grown in LUFA 2.2 soil for 21 d, after application of glyphosate, dimethoate and spirodiclofen (mean net response with standard error bars). All units (nominal values) in mg AI/Kg dry soil. (* indicates statistical differences (Dunnett´s method, $p<0.05$)).

Table 4.2: EC_{50} values (mg AI/Kg dry soil) and 95% confidence intervals (CI) for the effects of single exposure pesticides on the shoot length and fresh weight of *Triticum aestivum* exposed for 21 days on LUFA 2.2 soil. Values included were also derived from single chemical exposure in tests run simultaneously with the mixture exposure.

Pesticide	Shoot length (cm/plant)	Fresh weight (mg/plant)	Experiment
Glyphosate	156.9 (130.2 – 163.6)	129.4 (85.6 – 173.1)	Single exposure
	151.7 (107.4 – 196.0)	153.7 (102.6 – 194.7)	Mixture with dimethoate
	91.5 (5.6 – 177.5)	105.3 (28.5 – 182.2)	Mixture with spirodiclofen
Dimethoate	19.3 (12.7 – 25.9)	11.4 (1.2 – 20.8)	Single exposure
	22.9 (17.9 – 28.1)	22.0 (16.4 – 35.7)	Mixture with glyphosate
	10.5 (5.8 – 17.9)	12.3 (7.8 – 32.3)	Mixture with spirodiclofen
Spirodiclofen	2.4 (1.7 – 2.6)	2.5 (1.2 – 2.6)	Single exposure
	1.8 (1.5 – 2.4)	2.2. (1.5 – 2.5)	Mixture with glyphosate
	1.3 (0.7 – 2.3)	1.5 (1.3 – 2.0)	Mixture with dimethoate

Table 4.3: EC_{50} values (mg AI/Kg dry soil) and 95% confidence intervals (CI) for the effects of single exposure pesticides on the shoot length (SL) and fresh weight (FW) of *Brassica rapa* exposed for 21 days on LUFA 2.2 soil. Values included were also derived from single chemical exposure in tests run simultaneously with the mixture exposure.

Pesticide	Shoot length (cm/plant)	Fresh weight (mg/plant)	Experiment
Glyphosate	151.9 (122.1 – 171.7)	145.9 (73.6 – 161.5)	Single exposure
	147.3 (30.4 – 225.1)	97.3 (53.1 – 242.9)	Mixture with dimethoate
	144.4 (18.1 – 270.6)	90.9 (87.1 – 268.8)	Mixture with spirodiclofen
Dimethoate	35.8 (24.6 – 37.1)	28.8 (22.1 – 31.6)	Single exposure
	38.5 (22.3 – 39.1)	38.9 (31.1 – 39.9)	Mixture with glyphosate
	19.0 (8.3 – 24.5)	18.7 (15.8 – 23.5)	Mixture with spirodiclofen
Spirodiclofen	2.3 (0.3 – 2.6)	1.7 (0.3 – 2.5)	Single exposure
	1.9 (0.8 – 2.3)	1.3 (0.5 – 1.9)	Mixture with glyphosate
	2.1 (1.7 – 2.5)	2.4 (2.1 – 2.5)	Mixture with dimethoate

4.3.2 Mixture Toxicity

4.3.2.1. Glyphosate and spirodiclofen

After fitting the CA model to the SL data from the binary mixture of glyphosate and spirodiclofen, deviations from this reference model were observed in the two plant species. Antagonism described better the mixture for *T. aestivum* since when the parameter *a* was extended in the MIXTOX model the coefficient of determination (r^2) increased in comparison with the reference model, the residuals of the sum of squares (SS) decreased (Table 4.1; Table 4.4) and the p-value of the chi-test performed with the reference model was significant. When the IA model was fitted to the SL of *T. aestivum* the reference concept resulted as the best descriptive model to explain this mixture (Table 4.4); in this case adding parameters *a* and *b* did not improve the description of the mixture ($p > 0.05$), although there was a small reduction in the SS values. When the CA model was fitted to the SL of *B. rapa* a DL deviation was observed (Table 4.6), since adding parameters *a* and *b* improved the description of the data ($r^2 = 0.82$; SS = 38) in comparison with the reference model ($r^2 = 0.64$; SS = 75). Since parameter *a* was higher than zero and parameter *b* less than zero, this meant that antagonism occurs at low doses of the two pesticides and with high doses of the two pesticides synergism takes place. When the IA model was fitted to the SL data of *B. rapa* a deviation from the reference model was found, hence adding parameter *a* produced and improvement in the description of the data (Table 4.6). As parameter *a* was higher than zero, this means that antagonism occurred in the mixture, thus a smaller than expected toxicity was observed. Adding parameter *b* did not improve statistically the description of the data.

Fitting the CA model to the FW of *T. aestivum* and *B. rapa* after exposure to glyphosate and spirodiclofen resulted in an antagonistic deviation (Table 4.5; Table 4.7). Adding parameter *b* did not improve the description of the data for both plant species. When the IA model was fitted to the same data, the reference model itself was the most parsimonious model for both plants (Table 4.5; Table 4.7). This is an indication, that for this reference model, extending the MIXTOX with parameters *a* and *b* did not lead to a

more satisfactory description of the data, thus no interaction between the two pesticides was detected in this endpoint.

Table 4.4: Parameter estimates and tests of fit of the reference models using the MIXTOX model applied to the shoot length of *Triticum aestivum* exposed for 21d to three pesticide mixtures in LUFA 2.2 soil.

		Concentration Addition					Independent Action				
Mixture		r^2	p (χ^2)	SS	a	b	r^2	p (χ^2)	SS	a	b
Glyphosate	Reference	0.81	*	386	-	-	0.85	*	303	-	-
and	S/A	0.85	*	290	2.2	-	0.86	#	295	0.97	-
Spirodiclofen	DR	0.86	#	286	3.2	-1.9	0.85	#	294	1.9	-1.5
	DL	0.86	#	272	0.6	-1.6	0.86	#	280	0.0002	-809
Glyphosate	Reference	0.60	*	660	-	-	0.73	*	448	-	-
and	S/A	0.72	*	474	2.1	-	0.74	*	444	-0.83	-
Dimethoate	DR	0.79	*	347	5.4	-7.7	0.78	*	367	7.3	-15.8
	DL	0.73	#	453	0.1	-9.6	0.79	*	360	-6.5	1.6
Dimethoate	Reference	0.39	*	574	-	-	0.41	*	641	-	-
and	S/A	0.45	#	522	2.4	-	0.47	#	584	2.3	-
Spirodiclofen	DR	0.48	#	490	-1.9	9	0.54	*	499	-6.9	15.2
	DL	0.49	#	484	0.0006	-306.2	0.52	#	521	0.0054	-1679.2

r^2 is the coefficient of determination; p (χ^2) indicates the outcome of the likelihood ratio test (significance levels: * < 0.05; # > 0.05); SS is residuals sum of squares; *a* and *b* are the parameters of the deviations; S/A is synergism/antagonism, DR is dose-ratio deviations and DL is dose-level deviation from the reference models.

Table 4.5: Parameter estimates and tests of fit of the reference models using the MIXTOX model applied to the fresh weight of *Triticum aestivum* exposed for 21 d to three pesticide mixtures in LUFA 2.2 soil.

		Concentration Addition					Independent Action				
Mixture		r^2	p (χ^2)	SS	a	b	r^2	p (χ^2)	SS	a	b
Glyphosate	Reference	0.76	*	53095	-	-	0.82	*	39581	-	-
and	S/A	0.82	*	39908	1.9	-	0.82	#	39580	-0.03	-
Spirodiclofen	DR	0.83	#	36412	4.1	-4.5	0.83	#	37742	3.4	-5.9
	DL	0.82	#	39412	1.1	-0.5	0.86	#	280	0.0002	-809
Glyphosate	Reference	0.67	*	79619	-	-	0.76	*	57766	-	-
and	S/A	0.74	*	61286	-	-	0.76	#	56201	-1.5	-
Dimethoate	DR	0.80	*	46219	5.2	-7.5	0.81	*	46271	10.1	-26.5
	DL	0.75	#	58598	0.1	-10.2	0.82	*	42017	-7.7	1.5
Dimethoate	Reference	0.60	*	163684	-	-	0.67	*	136940	-	-
and	S/A	0.71	*	119685	3.1	-	0.70	*	120921	2.2	-
Spirodiclofen	DR	0.73	#	109105	-0.8	6.9	0.73	#	109320	-3.2	8.5
	DL	0.76	*	96293	0.07	-25.2	0.73	#	108936	0.008	-504.7

r^2 is the coefficient of determination; p (χ^2) indicates the outcome of the likelihood ratio test (significance levels: * <0.05; # >0.05); SS is residuals sum of squares; *a* and *b* are the parameters of the deviations; S/A is synergism/antagonism, DR is dose-ratio deviations and DL is dose-level deviation from the reference models.

Table 4.6: Parameter estimates and tests of fit of the reference models using the MIXTOX model applied to the shoot length of *Brassica rapa* exposed for 21 d to three pesticide mixtures in LUFA 2.2 soil.

		Concentration Addition					Independent Action				
Mixture		r^2	p (χ^2)	SS	a	b	r^2	p (χ^2)	SS	a	b
Glyphosate	Reference	0.64	*	75	-	-	0.78	*	46	-	-
and	S/A	0.73	*	59	1.9	-	0.82	*	37	1.6	-
Spirodiclofen	DR	0.74	#	56	3.5	-2.9	0.82	#	37	2.1	-0.9
	DL	0.82	*	38	1.5	-0.1	0.82	#	37	0.9	-1.4
Glyphosate	Reference	0.75	*	36	-	-	0.80	*	30	-	-
and	S/A	0.83	*	25	1.7	-	0.83	*	25	1.4	-
Dimethoate	DR	0.84	#	24	2.9	-2.2	0.83	#	25	1.9	-0.9
	DL	0.83	#	24	0.7	-1.2	0.83	#	25	0.1	-32.5
Dimethoate	Reference	0.61	*	119	-	-	0.70	*	91	-	-
and	S/A	0.79	*	64	3.4	-	0.81	*	60	3.2	-
Spirodiclofen	DR	0.79	#	64	3.4	-0.06	0.81	#	58	2.7	0.8
	DL	0.80	#	59	6.9	0.3	0.82	#	54	7.6	0.8

r^2 is the coefficient of determination; p (χ^2) indicates the outcome of the likelihood ratio test (significance levels: * <0.05; # >0.05); SS is residuals sum of squares; *a* and *b* are the parameters of the deviations; S/A is synergism/antagonism, DR is dose-ratio deviations and DL is dose-level deviation from the reference models.

Table 4.7: Parameter estimates and tests of fit of the reference models using the MIXTOX model applied to the fresh weight of *Brassica rapa* exposed for 21 d to three pesticide mixture in LUFA 2.2 soil.

		Concentration Addition					Independent Action				
Mixture		r^2	p (χ^2)	SS	a	b	r^2	p (χ^2)	SS	a	b
Glyphosate	Reference	0.81	*	66667	-	-	0.83	*	57008	-	-
and	S/A	0.84	*	55937	1.2	-	0.85	#	53285	1.0	-
Spirodiclofen	DR	0.86	#	50432	3.1	-3.5	0.85	#	51359	2.3	-3
	DL	0.84	#	55654	0.8	-0.4	0.85	#	53058	1.8	0.7
Glyphosate	Reference	0.82	*	42799	-	-	0.85	*	36951	-	-
and	S/A	0.85	*	35713	1.1	-	0.85	#	35887	0.7	-
Dimethoate	DR	0.86	#	32412	2.8	-3.1	0.86	#	34371	2.1	-3.3
	DL	0.85	#	34799	0.2	-2.9	0.85	#	35849	0.3	-2.1
Dimethoate	Reference	0.67	*	143835	-	-	0.75	*	110221	-	-
and	S/A	0.80	*	87829	3.2	-	0.82	*	77776	3.4	-
Spirodiclofen	DR	0.80	*	87818	3.1	0.2	0.83	#	77112	2.4	2.1
	DL	0.83	*	73083	9.9	0.3	0.87	*	56393	21.8	1.05

r^2 is the coefficient of determination; p (χ^2) indicates the outcome of the likelihood ratio test (significance levels: * <0.05; # >0.05); SS is residuals sum of squares; *a* and *b* are the parameters of the deviations; S/A is synergism/antagonism, DR is dose-ratio deviations and DL is dose-level deviation from the reference models.

4.3.2.2 Glyphosate and dimethoate

In the mixture of glyphosate and dimethoate, after fitting the CA and IA models to the SL of *T. aestivum* deviations from the reference model were observed (Table 4.4). A DR deviation was found to be the deviation that explained better the data after fitting CA, with a significant decrease in the SS and an increase in the variation that was explained. Since parameter *a* was higher than zero and parameter *b* smaller than zero, this meant that antagonism occurred when dimethoate was dominant in the mixture, and synergism occurred in mixtures where glyphosate was dominant (Table 4.1). DL was the deviation that explained better the data after fitting IA to data, with parameters *a* and *b* indicating that synergism occurs at low doses, but with increasing doses (higher than the EC_{50} level) antagonism starts to be observed (Table 4.1). For *B. rapa*, after fitting CA and IA models to the SL data, antagonism was the deviation found, since adding parameter *a* improved the model fit significantly (Table 4.6) with the parameter *a* smaller than zero, and a significant reduction in the SS values.

For the FW, after fitting the CA and IA models to *T. aestivum*, the same deviations to those encountered in the SL were observed. After fitting CA to the data a DR deviations was observed with parameters *a* and *b*, indicating that antagonism occurred in the mixture with the exception of mixtures where glyphosate was dominant (Table 4.5). After fitting the IA model, DL deviation indicated that synergism occurs at low doses, but with increasing doses (higher than the EC_{50} level) antagonism starts to be observed (Table 4.5). After fitting the CA model to the FW of *B. rapa*, antagonism was detected as the deviation that explained better our data (Table 4.7). When the reference model of IA was fitted, the reference model itself was the model that explained better the data, since subsequent addition of parameters to the MIXTOX did not bring significant improvements (Table 4.7).

4.3.2.3 Dimethoate and spirodiclofen

When the reference models were fitted to the SL data of the binary mixture performed with dimethoate and spirodiclofen, some deviations were detected. In *T. aestivum*, the CA reference model was the model that fitted better the data; although adding parameters *a* and *b* decreased the SS values, there was not a statistically significant improvement in describing the data (Table 4.4). After IA was fitted to the same data of *T. aestivum* a DR was observed, with parameters *a* and *b* indicating that synergism occurred in the mixture where spirodiclofen was dominant and antagonism occurred in mixtures where dimethoate was dominant (Table 4.1; Table 4.4). For *B. rapa*, antagonism was the deviation that explained better the data after fitting the CA and IA models (Table 4.5).

After fitting the CA model to the FW of *T. aestivum*, a DL deviation was observed, with parameter *a* and *b* showing that antagonism could be found at low doses and synergism at high doses of the two pesticides and that the magnitude of antagonism/synergism becomes dose level dependent (Table 4.5; Table 4.1). When the IA model was fitted to the same data, an antagonistic deviation was observed. After fitting the CA and IA models to the FW of *B. rapa* a DL deviation was found with parameters *a* and *b* indicating that that antagonism could be found at low doses and synergism only at high doses of the two pesticides and that the change between antagonism/synergism would occur at levels higher than the EC_{50} level (Table 4.1; Table 4.7).

4.4 Discussion

4.4.1 Single Exposure

4.4.1.1 Glyphosate

Glyphosate only caused effects in the growth pattern of wheat at high concentrations of the herbicide spiked in the soil. This could be explained by the fact that glyphosate is a designed post-emergence herbicide, usually applied at already developed plants. Glyphosate absorption takes place through the leaves from where it is translocated to the rest of the plant through the phloem, and finally reaches the target chloroplasts where it is responsible for inhibiting the EPSPS enzyme (Ribo, 1986). The EPSPS activity was evaluated in 1-14 day-old wheat seedlings growing in culture chambers and it was observed that during germination, one of the most important sources of enzyme was the seed scutellum, followed by the developed leaves where 43% of the whole stock of enzyme was encountered (Arnaud et al., 1994). In addition to the biochemical allocation of EPSPS, it should be pointed out that other studies had demonstrated that little or no inhibition of seed germination occurs in wheat species with preharvest glyphosate application (Yenish and Young, 2000). Previous studies with glyphosate showed that at labeled recommended rates this herbicide did not affect the growth and biomass of wheat (Soltani et al., 2009). Furthermore, glyphosate applied 1 to 31 d before seedling did not affect wheat yields or grain test weights (Ogg and Young, 1991).

In a recent study dealing with the effects of herbicide drift to *B. rapa*, it was concluded that concentrations below the field application rate did cause an effect in seed production but not in the shoot dry weight of this species (Olszyk et al., 2010). Another study dealing with *Brassica napus* L. demonstrated that even in the highest dose not all the plants died as a consequence of herbicide application (Jong and Haes, 2001). Similar results regarding the absence of toxic effects at labeled rates of glyphosate on the shoot growth and shoot dry weigh of *B. napus* were observed (Blackburn and Boutin, 2003; Petersen et al., 2007) . Again, it is pertinent to say that glyphosate is also more toxic when

plants are fully developed thus having large contact surfaces for penetration of the herbicide that can be translocated into the storage organs (Dekker and Chandler, 1985), thus it should be expected that absorption from the soil, where this herbicide was spiked, could represent lower toxicity than foliar application. From the above, it can be stated that the effects of the post-emergence herbicide glyphosate will necessarily depend on factors such as the development stage of the plant, the target species and the amount of active ingredient that is used (Doublet et al., 2009).

4.4.1.2 Dimethoate

The effects of the insecticide dimethoate were only observed at concentrations higher than should be expected in the soil, since the field application dose did not cause an impact in both endpoints evaluated. In a previous study the application of this systemic organophosphorous insecticide decreased chlorophyll, sugars and carbohydrates and total protein in wheat plants has been observed only after a growing period of two months (Abo-El-Seoud and Frost, 1998). A recent study had demonstrated that this species was not affected in the germination capacity but the mean dry weight of the seedlings decreased (Hanley and Whiting, 2005). Again it seems pertinent to state that dimethoate is used to control foliar insects, and it is applied to already grown plants, thus it should be expected, at it was said about glyphosate, that absorption through soil should be less toxic to plants.

The effects of dimethoate to *B. rapa* were observed only at high concentrations of the systemic insecticide dimethoate. A previous study has evaluated the effect of the influence of leaf wax cover of *B. napus*, on the bioavailability of dimethoate to the collembolan *Folsomia candida* Willem (Chowdhury et al., 2005), and concluded that no effects were observed in the leaves of grown *B. napus* due to the foliar application of this insecticide. Previous studies had observed that dimethoate decreased oil content in the developing seeds of Indian mustard (*Brassica juncea* L.) but showed an increase in the mature seeds (Munshi et al., 1987) although these effects were not impairment to the growth of this Brassicaceae species. It can be concluded that this insecticide, if applied at recommended doses, does not exert a toxic effect on the germination potential and vegetative vigour *of B. rapa*.

4.4.1.3 Spirodiclofen

Spirodiclofen had no effects at the labeled dose of *T. aestivum* and *B. rapa*. There are several reports about the effects of spirodiclofen in target pests and no effects on plants where this acaricide was applied were observed (Hardman et al., 2003; Raudomis, 2006). According to the reports about the distribution and metabolism of spirodiclofen in apples, citrus and grapes, sprayed at fruit setting and before harvesting, using the commercial formulation of this acaricide, found no evidence of metabolic or biochemical effects in the referred species (Koester, 2002). In addition, a report about the ecobiological profile of spirodiclofen (Wachendorff et al., 2002; Nauen, 2005) concluded that no effects in terrestrial plants were to be anticipated if this PPP was to be applied at field rates. The present study seems to corroborate these previous findings, since differences in the growth of wheat were only observed at concentrations higher than should be expected if this acaricide is applied at labeled doses in the field. The fact that no effects on *T. aestivum* and *B. rapa* growth and biomass were observed at field recommended dose seems to corroborate the previous observations on the absence of detrimental effects at labeled doses of this acaricide.

4.4.2 Mixture Exposure

Although single exposures of the three PPPs used provided information that corroborates with already available data, the approach of single evaluations had to be carried out to proceed for the mixture toxicity evaluation.

4.4.2.1 Glyphosate and spirodiclofen

Fitting the CA model to the SL and FW data from the two plant species obtained resulted in an overall antagonistic deviation. There was conformity in the antagonistic deviation produced by the binary combination of glyphosate and spirodiclofen, only in the length of *B. rapa* where the outcome was a DL deviation, although with the prediction that antagonism would be encountered in low doses of the two pesticides. As a consequence the growth rate of wheat exposed to low concentrations of the two PPPs had a smaller impact

in the growth ability of this species than it was expected from the single exposures. This may be due to the fact that when the pesticides were applied jointly at sublethal concentrations, the plants were not affected in their vegetative vigour and the decrease in length and FW was only observed in the highest doses of each pesticide. A previous work where wheat was exposed to new aminoalkane and aminofluorenephosphonates compounds in binary mixture with glyphosate at concentrations around the respective EC_{50}, reported that the interaction between the mixture were mainly antagonistic (Bielecki et al., 2004). This antagonistic deviation can be associated with processes resulting from interactions in the medium (pesticide-pesticide binding in soil), interactions at site of uptake, and interactions at the target site (one pesticide may affect the binding of the other to the target cell), which could be the explanation for the reduced toxicity in some pesticide mixtures (Spurgeon et al., 2010).

When the IA model was fitted to the same data, this reference model was the most parsimonious model explaining the joint effects of these PPPs with the exception of the length of *B. rapa*, where antagonism was observed (the same data that rendered a different outcome when CA was chosen as a reference model). Nevertheless it can be said that again there was conformity in the outcome of fitting this reference model to both endpoints and plant species, which seem to indicate that these pesticides acted independently according to their expected different MoA (Greco et al., 1995).

4.4.2.2 Glyphosate and dimethoate

After fitting the CA and IA models to the SL and FW of the mixture of glyphosate and dimethoate, deviations from the reference models were observed for *T. aestivum*. When the CA model was fitted to data a DR deviation was observed, meaning that antagonism occurs in the mixture with the exception for the combinations where the toxicity was caused mainly by glyphosate. In the combinations where this herbicide was the dominant toxicant, synergism took place, meaning more toxicity than it was expected. After fitting the IA model a DL deviation was derived, with synergism at low doses and a change to antagonism at doses higher than the EC_{50} level.

The apparent disagreement in predicting antagonism (DR deviation) or synergism (DL deviation) should be addressed taking in consideration the real data observed and comparing it with the two opposed deviations derived from the MIXTOX. This will allow us to compare the two deviations and choose the "best" descriptive deviation of the data. In order to do this we plotted the length and FW data and the two deviations (DR after CA fit and DL after IA fit) following the logarithm of the toxic units of each binary mixture performed (Fig. 4.4 and Fig. 4.5). After analyzing the graphs it can be concluded that DL (with synergism at low doses and antagonism only at doses higher than the EC_{50} level) provided the best description of the data, since the line plotted fitted the data points of the observed values better than the DR line. In conclusion, synergism predicted at low doses of the two pesticides and antagonism at high concentrations (and therefore with less biological significance) seems to be the pattern that should be derived from the interaction of these two pesticides.

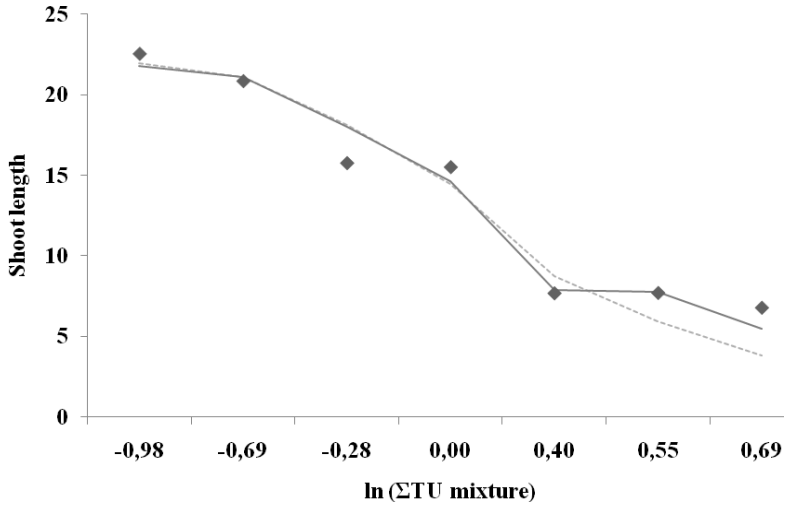

Fig. 4.4 - Effect of glyphosate and dimethoate (ln ∑TU) on the shoot length of *Triticum aestivum* (cm per plantl). Observed values from binary mixtures (square dots), modeled dose-response values after fitting the dose ratio from concentration addition (dotted line) and after fitting the dose level-dependent deviation from independent action (straight line).

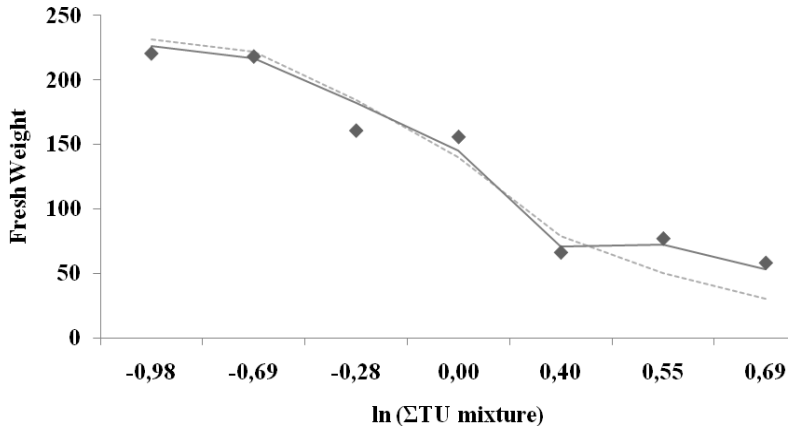

Fig. 4.5 - Effects of glyphosate and dimethoate (ln ∑TU) on the fresh weight of *Triticum aestivum* (mg per plant). Observed values from binary mixtures (square dots), modeled dose-response values after fitting the dose ratio from concentration addition (dotted line) and after fitting the dose level-dependent deviation from independent action (straight line)

Organophosphorous insecticides, like dimethoate, are known to inhibit the cytochrome P450 in terrestrial plants (Biediger et al., 1992) which is know to play an important role in detoxification processes of several xenobiotic in plants (Munkegaard et al., 2008). It also has been described that glyphosate inhibits the cytochrome P450 enzyme of wheat plants, which will increase the proportion of toxic molecules reaching the target site (Xiang et al., 2005). Thus, a possible explanation for the synergism detected in this mixture could be the joint effect of the two stressors in biochemical impairment of cytochrome P450.

After fitting the CA and IA models to the FW and length of *B. rapa* deviations were also found. When the CA model was fitted to the length and FW data, antagonism was the deviation observed, thus it can be said that a lower toxicity than expected was observed in both endpoints. A significantly lower toxicity was also observed in a mixture of glyphosate and the organophosphorous insecticide chlorpyrifos applied at field recommended doses to *B. napus* (Martin and Ronco, 2006). When the IA model was fitted to SL data antagonism was observed. Antagonism was also detected in a work dealing with the joint effects of organophosphorous insecticides and herbicides (metsulfuron-methyl, terbutylazine and

bentazone) on the green algae *Pseudokirchneriella subcapitata* and aquatic plant *Lemna minor* (Munkegaard et al., 2008). In the cited work it was conclude that the majority of mixtures performed resulted in antagonism, and no synergism (enhanced toxicity) was registered. The only exception to this general antagonistic pattern was when the IA model was fitted to the FW data, whereas the reference model itself was the most parsimonious model for explaining the mixture toxicity. The outcome was that the pesticides acted independently, meaning that the susceptibility to glyphosate may or may not be correlated with the susceptibility to dimethoate (Wilbur et al., 2004).

4.4.2.3 Dimethoate and spirodiclofen

When the CA model was fitted to the SL data of *T. aestivum* the mixture with dimethoate and spirodiclofen the reference model itself was the most parsimonious model. According to the CA model the resulting toxicity of the mixture can be accounted as a simple summation of the toxicity of each pesticide present in the mixture (Loureiro et al., 2010). But when the IA reference model was fitted to the same data a DR deviation was detected, with synergism occurring when spirodiclofen was dominant in the mixtures and antagonism when dimethoate was dominant in the mixtures. When the CA model was fitted to the FW, a DL deviation was observed with a magnitude of antagonism dose level dependent (Jonker et al., 2005), and when IA was fitted to the same data, antagonism was again found as the deviation that explained better the interaction between these two pesticides. The difference between the two endpoints following CA and IA fitting of the data, seemed to indicate that in this particular case, the choice of endpoint led to different predictions, although not contradictory. Previous studies have already demonstrated that the toxic effects could vary according to the endpoint evaluated (Turgut and Formin, 2002). Thus it has been recognized that the choice of endpoint could lead to different assumptions in mixture toxicity experiments (Cedergreen and Streibig, 2005).

For the combination of dimethoate and spirodiclofen, antagonism was the general pattern observed after fitting CA and IA models to the SL and FW data of *B. rapa*. After fitting the CA and IA models to the length data, antagonism was the deviation that better explained the data, but when these models were fitted to the FW a DL deviation indicating

that antagonism would be expected unless the doses of the two pesticides were above the EC_{50} level. Antagonism has also been detected in binary mixtures applied to *Brassica* plants, and the reason appointed to the resultant toxic effects being smaller than expected were the competition in terms of the pesticide intake by the plants (An and Lee, 2007). In the present study one cannot exclude the possibility of chemical interaction between these two insecticides in the soil, and the concomitant lesser absorption of chemical by the plants via the soil matrix, since it has been described that the soil represents a good basis for interaction between substances, which could reduce their bioavailability to terrestrial plants (Matzke et al., 2008). Other interactions between these two pesticides could also mitigate the effects observed when the pesticides are applied in the mixture exposures in comparison with the effects observed in the single exposures performed.

4.4.3 Comparing species sensitivity to the PPPs

In an overall look about the results in single exposure toxicity tests, it could be stated that the EC_{50} obtained in the single toxicity experiments and in the single experiments that ran simultaneously with the mixture experiments were in the same range interval for both test species (Table 4.2, Table 4.3). The only exception was the insecticide dimethoate, that was slightly more toxic to *T. aestivum* than to *B. rapa* in the two endpoints evaluated, although the difference in the EC_{50} calculated varied only by a factor of two. Thus, it can be said that regarding the vegetative vigour of these species, no significant differences in their sensitivity to the single exposure to three PPPs were observed.

From the analyses of the several binary mixtures performed, regarding the effects on the SL and FW, there was one combination (glyphosate and dimethoate applied to *T. aestivum*) where the prediction observed after fitting the CA model was apparently incongruent with the prediction made when the IA model was fitted to the same data. In fact, after modelling the values in the CA model a DL deviation with antagonism at low doses was observed, whereas the prediction after modelling the data with IA model derived a DR deviation with antagonism except for the mixtures where glyphosate was dominant in the mixture. Given that the herbicide glyphosate and the insecticide dimethoate have different MoA, and it has been demonstrated that the predictability of the IA model could

be more adequate for evaluating the interaction between two pesticides with differentiate MoA, maybe this could be one reason for why after fitting IA to the data a better description of the data could be established (Loureiro et al., 2009).

4.5 Conclusions

Only at high concentration of the three pesticides, changes in the vegetative vigour (shoot length and fresh weight) of *T. aestivum* and *B. rapa* were detected. As a result, at the field recommended doses no detrimental effects would be anticipated in the growing ability of the two plant species. After fitting the CA and IA model to the data of SL and FW of the two plants, antagonism was observed in 16 of the 24 test-trials, the two conceptual models were the most parsimonious models in 5 of the 24 test-trials and only in 3 test-trials the resulting interaction was synergistic at low doses of the two pesticides and changes to antagonism with increasing doses of the pesticides. Since synergism is the worrying condition in mixture toxicity assessment, it could be concluded that, apparently, mixing and applying these PPPs at recommended doses did lead to an increase in toxicity, the exception was the binary mixture of glyphosate and dimethoate applied to *T. aestivum*. There was a general agreement in terms of the outcome of fitting the models to the endpoints in the two plant species, implying that when submitted to the same binary mixture similar conclusions could be derived from the two species.

References

Abo-El-Seod, M.A., Frost, M., 1998. Biochemical changes in wheat plants as affected by residues of dimethoate and pirimicarb. Environmental Management and Health, 9(5), 188-193.

An, Y.-J-., Lee, W.-M., 2007. Decreased toxicity to terrestrial plants associated with a mixture of methyl *tert*-butyl ether and its metabolite *tert*-butyl alcohol. Environmental Toxicology and Chemistry 26 (8), 1711-1716.

Andersen, M. E., Dennison, J. E., 2004. Mechanistic approaches for mixture risk assessments – present capabilities with simple mixtures and future directions. Environ. Toxicol. Pharm. 16, 1–11.

Arnaud, L., Nurit, F., Ravanel, P., Tissut, M., 1994. Distribution of glyphosate and of its target enzyme inside wheat plants. Pesticide Science 40 (3), 217-223.

Biediger, D.L., Baumann, P.A., Weaver, D.N., Chandler, J.M., Merkle, M.G., 1992. Interactions between primisulfuron and selected soil-applied insecticides in corn (*Zea mays*). Weed Technol 6, 807–812.

Bielecki, K., Sarapuk, J., Kleszczyńska,H., 2004. Binary mixtures of (*n*-phosphonomethyl)-glycine with new aminophosphonates. Z. Naturforsch. 59c, 515-518.

Blackburn, L.G., Boutin, C., 2003. Subtle effects of herbicide use in the context of genetically modified crops: a case study with glyphosate (Roundup). Ecotoxicology 12, 271-285.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 585–615.

Cedergreen, N., Streibig, J.C., 2005. Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? Environmental Toxicology and Chemistry 24(7), 1676-1683.

Chopra, S.I., Nandra, K.S., 1969. Effect of thiometon on the germination of sarson (*Brassica campestris* Linn. Var Brown sarson). Journal of Agricultural and Food Chemistry, 17, 805-806.

Chowdhury, A. B. M. N. U., Jepson, P.J., Ford, M.G., Frampton, G.K., 2005. The role of cuticular waxes and surface roughness in determining the insecticidal efficacy of deltamethrin and dimethoate applied as emulsifiable concentrates to leaf surfaces. International Journal of Pest Management, 51(4): 253 – 263.

Dekker, J.H., Chandler, K., 1985. Herbicide effect on the viability of quackgrass (*Agropyron repens*) rhizome buds. Can. J. Plant Sci. 65, 1057–64.

Doublet, J., Mamy, L., Bariuso, E., 2009. Delayed degradation in soil of foliar herbicides glyphosate and

sulcotrione previously absorbed by plants: consequences on herbicide fate and risk assessment. Chemosphere 77, 582-589.

Duke, S.O., Bowles, S.B., 2008. Glyphosate: a once-in-a-century herbicide. Pest Management Science 64, 319-325.

Egley, G.H., Williams, R.D., 1978. Glyphosate and paraquat effects on weed seed germination and seedling emergence. Weed Science, 26 (3), 249-251.

Eurostat, 2007. The use of plant protection products in the European Union. Data 1992-2003. Eurostat Statistical books, 2007 edition.

Gange, A.C., Brown, V.K., Farmer, L.M., 1992. Effects of pesticides on the germination of weed seeds: implications for manipulated experiments. Journal of Applied Ecology, 29: 3030-310**.**

GCPF (Global Crop Protection Federation), 2000. A comparative review of terrestrial (non-target) plant test methods. OECD Background document.

Greco, W., Unkelbach, H.-D., Pöch, G., Sühnel, J., Kundi, M., and Bodeker, W., 1992. Consensus on concepts and terminology for combined-action assessment: The Saariselkä agreement. Arch. Complex Environ. Studies 4, 65–69.

Greco, W.R., Bravo, G., Parsons, J.C., 1995. The search for synergy: A critical review from a response durface perspective. Pharmacological Review 47, 332-385.

Hardman, J.M., Franklin, J.L., Moreau, D.L., Bostanian, N.J., 2003. An index for selective toxicity of miticides to phytophagous mites and their predators on orchard trials. Pest Management Science, 59, 1324-1332.

ISO (International Organization for Standardization), 1995. Soil quality – Determination of the effects of pollutants on soil flora – Part 2: Effects of chemicals on the emergence and growth of higher plants. ISO/DIS 11269-2. Geneve, Switzerland.

ISO (International Organization for Standardization), 2004. Soil quality – Biological methods – Chronic toxicity in higher plants. ISO/DIS 22030, Geneve, Switzerland.

Jong, F.M.W., Snoob, G.R., van de Zande, J.C., 2008. Estimated nationwide effects of pesticide spray drift on terrestrial habitats in the Netherlands. Journal of Environmental Management 86: 721–730.

Jong, F.M.W., Haes, H.A.U., 2001. Development of a field bioassay for the side-effects of herbicides on vascular plants using *Brassica napus* and *Poa annua*. Environmental Toxicology, 16, 397-407.

Kalsch, W., Junker, T., Römbke, J., 2006. A chronic plant test for the assessment of contaminated soils. Part 1: Method development. J Soils & Sediments 6 (1), 37 – 45.

Klingman, D.L., Murray, J.J., 1976. Germination of seeds of turfgrasses as affected by glyphosate and paraquat. Weed Science 24 (2), 191 – 193.

Koester, J., 2002. Metabolism of spirodiclofen (BAJ 2740) in animals and plants. Bayer Crop Sci J. 55, (2/3), 237-254.

Loewe, S., Muischnek, H., 1926. Effect of combinations: mathematical basis of problem. N-S. Arch. Ex. Path. Ph. 114, 313–326.

Long, S.M., Reicheinberg, F., Lister, L.J., Hankard, P.M., Townsend, J., Mayer, P., Wright, J., Holmstrup, M., Svendsen, C:, Spurgeon, D.J., 2009. Combined chemical (fluoranthene) and drought effects on Lumbricus rubellus demonstrate the applicability of the independent action model for multiple stressor assessment. Environmental Toxicology and Chemistry 28 (3), 629-636.

Loureiro, S., Amorim, M.J.B., Campos, B., Rodrigues, S.M.G., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157 (2), 625–632.

Loureiro, S., Svendsen, C., Ferreira, A.L.G., Pinheiro, C., Ribeiro, F., Soares, A.M.V.M., 2010. Toxicity of three binary mixtures to Daphnia magna: comparing chemicals mode of action and deviations from conceptual models. Environmental Toxicology and Chemistry doi: 10.1002/etc.198

Martin, M. L., Ronco, A. E. , 2006. Effect of mixtures of pesticides used in the direct seeding technique on nontarget plant seeds. Bull. Environ. Contam. Toxicol., 77:228–236.

Matzke, M., Stolte, S., Böschen, A., Filser, J., 2008. Mixture effects and predictability of combination effects in imidazolium based ionic liquids as well as imidazolium based ionic liquids and cadmium on terrestrial plants (*Triticum aestivum*) and limnic green algae (*Scenedesmus vacuolatus*). Green Chemistry 10, 784-792.

Munkegaard, M., Abbaspoor, M., Cedergreen, N., 2008. Organophosphorous insecticides as herbicide synergists on the green algae *Pseudokirchneriella subcapitata* and the aquatic plant *Lemna minor*. Ecotoxicology 17, 29-35.

Munshi, S.K., Kumari, A., Sanadi, S., Bakhetia, D.R.C., Sukhija, P.S., 1987. Influence of systemic insecticides on lipid biosynthesis in seeds of Indian mustard (*Brassica juncea* L.). Plant Science, 53 (3), 201-208.

Nauen, R., 2005. Spirodiclofen: mode of action and resistance risk assessment in Tetranychid pest mites. J. Pestic. Sci. 30, 272–274.

Ogg, Jr., A.G., Young, F.L., 1991. Effects of preplant treatment interval and tillages on herbicide toxicity to winter wheat (*Triticum aestivum*). Weed Technology, 5 (2), 291-296.

Olszyk, D., Pfleeger, T., Lee, H.E., Plocher, M., 2010. Phytotoxicity assay for seed production using *Brassica rapa* L. Integrated Environmental Assessment and Management. DOI: 10.1002/ieam.89

Petersen, I.L., Hansen, H.C.B., Ravn, H.W., Sørensen, J.C., Sørensen, H., 2007. Metabolic effects in rapeseed (*Brassica napus*, L.) seedlings after root exposure to glyphosate. Pesticide Biochemistry and Phisiology 89, 220-229.

Raudomis, L., 2006. Comparative toxicity of spirodiclofen and lambdacihalotrin to *Tetranychus urticae*, *Tarsonemus pallidus* and predatory mite *Amblyseius andersoni* in a strawberry site under field conditions. Agron. Res. 4, 317-322.

Ribo, J.M., 1986. ‘‘Roundup’’ (Glyphosate): A TRC Technical Review. Saskatchewan: Toxicology Research Centre, University of Saskatchewan.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2010. Joint effects of three plant protection products to the terrestrial isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. Chemosphere 80, 1021-1030.

Soltani, N., Shropshire, C., Sikkema, P.H., 2009. Sensitivity of winter wheat to preplant and preemergence glyphosate tankmixes. Crop Protection, 28 (5), 449-452.

Song, N.H., Yin, X., L., Chen G.F., Yang, H., 2007. Biological responses of wheat (*Triticum aestivum*) plants to the herbicide chlorotoluron in soils. Chemosphere 68, 1779–1787.

Spurgeon D.J., et al, Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures, Sci Total Environ (2010), doi:10.1016/j.scitotenv.2010.02.038

Steinrucken, H. C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolylpyruvyl-shikimic acid-3-phosphate. Biochem. Biophys. Res. Comm. 94:1207–1212.

Turgut, C., Formin, A., 2002. Sensitivity of the rooted macrophyte *Myryophyllum aquaticum* (Vell.) Verdcourt to seventeen pesticides determined on the basis of EC_{50}. Bulletin of Environmental Contamination and Toxicology, 69, 601-608.

Wachendorff, U., Nauen, R., Schnorbach, H.J., Rauch, N., Elbert, A., 2002. The biological profile of Spirodiclofen (Envidor®) – a new selective tetronic acid acaricide. Bayer Crop Sci J. 73 (55) 149–176.

Wilbur, S.B., Hansen, H., Pohl, H., Colman, J., McClure, P., 2004. Using the ASTDR guidance manual for the assessment of joint toxic action of chemical mixtures. Environmental Toxicology and Chemistry 18, 223-230.

Wolf, C., Schnorbach, H.-J., 2002. Ecobiological profile of the acaricide spirodiclofen. Bayer Crop Sci J. 55 (3), 177-906.

Xiang, W.-S., Wang, X.-J., Ren, T.-R., Ju, X.-L., 2005. Expression of a wheat cytochrome P450 monooxygenase in yeast and its inhibition by glyphosate. Pest Management Science 61, 402-406.

Yang, R.S.H., 1994. Introduction to the toxicology of chemical mixtures. In Yang, R.S.H., ed, Toxicology of Chemical Mixtures. Academic, San Diego, Ca, USA, pp 1-10.

Yenish, J.P, Young, F., 2000. Effect of preharvest glyphosate application on seed and seedling quality of spring wheat (*Triticum aestivum*). Weed Technology 14, 212-217.

Zwart, D., Posthuma, L., 2005. Complex mixture toxicity for single and multiple species: proposed methodologies. Environmental Toxicology and Chemistry 24 (10), 2665-267.

5. EVALUATION OF THE JOINT EFFECT OF DIMETHOATE AND SPIRODICLOFEN TO PLANTS AND EARTHWORMS IN A DESIGNED MICROCOSM EXPERIMENT

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Abstract

Most studies in soil ecotoxicology imply testing the effects on just one chemical in one test species using a reference soil (e.g. LUFA 2.2, OECD soil). There is an urgent need for a more comprehensive, holistic, hierarchical and integrated approach to assess the impacts of chemical pollutants on soil ecosystems. An attempt to accomplish an higher "realism" in ecotoxicity evaluation was made by designing a microcosm experiment, using a small-scale terrestrial ecosystem ("STEM") containing a Mediterranean agricultural soil, where earthworms (*Eisenia andrei*) and turnip seeds (*Brassica rapa*) were incorporated to survey the effects of the acaricide spirodiclofen and the insecticide dimethoate in single exposure along with binary combinations of the two pesticides. The results showed that for plants and earthworms the recommended application dose of both pesticides did not cause any impairment in the growth pattern of both test species, although with increasing concentrations a trend in decrease biomass could be observed for both test species. Earthworms were also sensitive in their depth distribution due to the application of the two pesticides, and this parameter was more sensitive to pesticide application than the variation in body weight. The several binary mixtures performed showed that according to the independent action model there was antagonism (smaller effect than expected) for the *B. rapa* parameters (shoot length and fresh weight). Regarding the depth distribution of *E. andrei* in the field dose and five times the field dose of the two pesticides, antagonism was observed whereas in the highest concentrations tested there was a synergistic pattern, i.e. worms escaped more than predicted by the IA model.

5.1 Introduction

Environmental toxicology has been focusing mainly the assessment of single toxic pollutants to single tests organisms, as it is established in several guidelines available for ecotoxicological studies (e.g. ISO, OECD). However, to bridge the gap between laboratorial single species tests, often criticized in terms of mode of exposure to a pollutant and for the problems in deriving and extending results to what happens in the field, and field studies, microcosms have been proposed as a good alternative for testing the effects of pollutants (Edwards, 2002).

A terrestrial microcosm can be defined as a soil unit consisting either in soil taken from the field and sieved or intact soil cores where several species are introduced in order to assess the impacts of pollutants at different levels of biological organization (Burrows and Edwards, 2002) Environmental risk assessment is usually made by evaluating the effects of one single chemical to one test-species, and results are then extrapolated to the community level (De Laender *et al.*, 2009), but the effects on the entire diversity of organisms in the edaphic ecosystems could be underestimated in single-species testing (Edwards, 2002), thus microcosms may lead to more realistic and sensitive results (Alonso *et al.*, 2009).

Pesticides sprayed in agricultural environments may impact both target species and non-target organisms or bordering areas (Reinecke and Reinecke, 2007). Earthworms are among these non-target organisms, and are of paramount importance to edaphic ecosystems, due to their important role in soil formation and structure through consumption of dead plant and animal matter, mixing of particles during digestion, cast deposition throughout the soil column, improvement of aeration and drainage of the soil by burrowing (Lydy and Linck, 2003). Non-target plants in off-crop areas can be affected by indirect drift of the products applied to control weeds and pests (Jong *et al.*, 2008). Therefore, it is important to assess the potential pernicious effects that the application of pesticides may have in non-target terrestrial plants.

Dimethoate acts through the inhibition of acetylcholinesterase (AChE) enzyme activity in arthropods (Martikainen, 1996); its mode of action in plants has been shown to

be internal to the seeds through the inhibition of the synthesis or action of hydrolytic enzymes (Gange *et al.*, 1992). Spirodiclofen is a selective, non-systemic acaricide, and the risks to terrestrial plants have not been anticipated due to its specific mode of action as a lipid biogenesis inhibitor in invertebrates (Wolf and Schnorbach, 2002). The two plant protection products (PPPs) were chosen based on their different modes of action, but also due to the market share that dimethoate has in Europe as the one of the most sold insecticides and spirodiclofen, which has been recently introduced in Portugal (Vieira, 2009).

It is recognized that contamination problems are often characterized by complex mixtures of chemicals (Loureiro *et al.*, 2009). The Independent Action (IA) reference model has been developed for chemicals with different mode of action and assumes that the effects of components in the mixture are statistically independent, thus the probability of the effect of one chemical is independent from the probability of the effect of the second chemical in the mixture (Loureiro *et al.*, 2010). However, sometimes compounds can interact synergistically or antagonistically resulting in a stronger or lesser toxicity than it should be expected (Cassee et al., 1999).

The objectives of this work were firstly to design a microcosm experiment which could mimic what happens in real exposure scenarios after the application of PPPs, and secondly to evaluate the combined effects of two pesticides to the earthworm *Eisenia andrei* and the turnip species *Brassica rapa* using the concept of independent action.

5.2 Materials and methods

5.2.1 Test organisms and test soil

Earthworms of the epigeic species *Eisenia andrei* (Bouché), bought from a commercial supplier and seeds of *Brassica rapa* (L.) rapid cycle (Carolina Biological Supply Company) were used in the experimental procedure.

An agricultural soil was collected in the spring of 2010 (first 20 cm of soil collected) from the lower Mondego Valley (Portugal). The soil was brought from an agricultural field that has not been pesticide-treated in the last five years (Lemos *et al.*, 2010). Soil parameters included a pH (H_2O) = 7.48, organic matter content = 2.4 %, clay = 4.2 %, silt = 7.0%, sand = 88.7%, density (g/cm^3) = 2.4 and a water holding capacity = 70%. The soil was brought to the laboratory, sieved (5 mm) and air dried prior to experiment.

5.2.2 Test chemicals

The effects of dimethoate and spirodiclofen on test species were studied using three different concentrations, ranging from the field dose (FD), i.e. the recommended application rate according to the label of the commercial formulation of the product, to 10 times the FD of the of dimethoate (Agror®) and spirodiclofen (Envidor®).

More specifically, the nominal concentrations used for dimethoate ranged from 0.3 mg active ingredient (a.i.)/Kg dry soil (recommended labeled dose), 1.5 mg a.i./Kg dry soil (5 fold the recommended dose) and 3 mg a.i./Kg dry soil (10 fold the recommended dose); for spirodiclofen the nominal concentrations ranged from 0.06 mg a.i./Kg dry soil (recommended labeled dose), 0.3 mg a.i/Kg dry soil (5 fold the recommended dose) and 0.6 mg a.i/Kg dry soil of spirodiclofen (10 fold the recommended dose). The binary mixtures were made using the same concentrations of the two pesticides at the field dose, 5 times the FD and 10 times the FD in a one fixed ratio design (Fig. 5.1). For each treatment and control, three replicates were made, in a total of 30 microcosms tested. Pesticides were applied at the surface of the soil layer using a common sprayer.

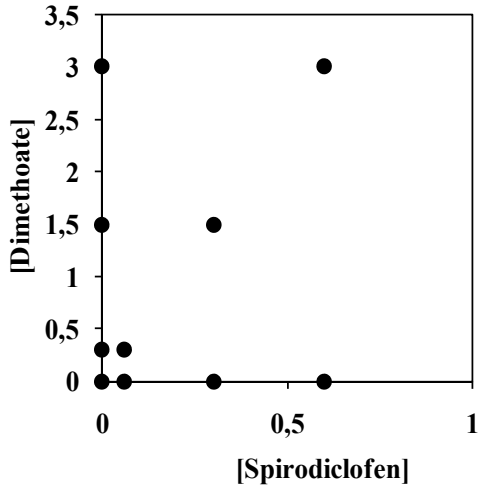

Fig. 5.1 - Experimental design of the binary combinations performed, based on the concentrations of dimethoate and spirodiclofen (all units are in mg a.i./Kg soil).

5.2.3 Small-scale terrestrial ecosystem (STEM)

The small-scale terrestrial ecosystems (STEM) consisted on 2mm wall cylindrical PVC pipes (12 cm diameter and 38 cm deep) with a surface area of 0.095 m^2, filled to within 5 cm of the top with agricultural soil (Fig. 5.2). Each STEM was filled with approximately 4 kg of soil. The bottom of the microcosms was covered with fine plastic gauze (1.0 mm aperture) to avoid earthworms' escaping from the microcosms (Fig. 5.2).

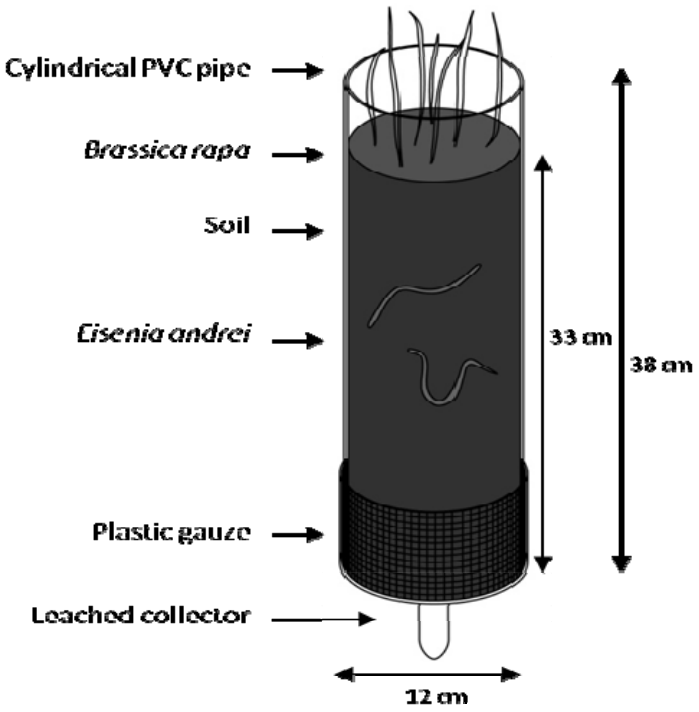

Fig. 5.2 - Small-scale terrestrial ecosystem (STEM) used in the experimental procedure.

The STEM were placed in an acclimatized moveable cart (83 cm length * 55 cm width * 55 cm depth) with the lid hollowed in order to enclose 5 STEM (Fig. 5.3). These acclimatized chambers allowed to maintain soil temperature (12 º C) and moisture of the STEM in controlled conditions (Fig. 5.4). Six acclimatized chambers, each one with 5 STEM, were used in the experimental procedure.

Fig. 5.3 - View of one acclimatized moveable cart under laboratory conditions.

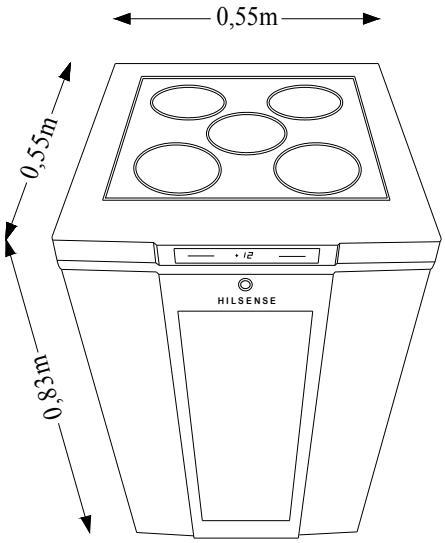

Fig. 5.4 - Schematic view of the acclimatized moveable cart with the enclose STEM.

5.2.4 Experimental Setup in STEM

Each small-scale terrestrial ecosystem (STEM) was incubated in an experimental room at 20 ± 2º C, with a 16/8 h light dark regime for a period of 28 days. Soil water content was adjusted to 70% of maximum water holding capacity (WHC), making it comparable to field moisture, and was subsequently maintained throughout the experiment by adding sterile water in order to simulate rainfall in spring normal conditions (≈ 84 mm per day). Ten adult clitellate earthworms and ten turnip seeds were introduced on soil surface of each microcosm. Earthworm weight was recorded (as a group) before their introduction into the microcosms. At the end of the 28 d exposure period the fresh weight and shoot length of *B. rapa* were measured as well as the fresh weight and depth distribution of *E. andrei* along the microcosms.

5.2.5 Chemical analysis

The chemical analysis was performed after 28 days of exposure. Soil samples from the upper soil layer (0 - 10 cm) and the lower soil layer (10 - 20 cm) were taken and sent for chemical analysis at Marchwood Scientific Services, Southampton, UK. The method used for the analysis of the soil samples with dimethoate and spirodiclofen was as follows: soil samples were first air dried and ground and a 1-2 gram sample was extracted with acidified acetonitrile. The sample was then filtered and the filtrate subjected to further analysis by Liquid Chromatography-tandem Mass Spectrometry following a pre-treatment buffering stage. The instrument used for the analysis was an Agilent 6410 Triple quad LCMS-MS. Standards were prepared in solvents at 7 levels with recoveries in the range 80-120%

5.2.6 Statistical analysis

Differences among plants and earthworms with PPP application and their respective controls were analyzed using a one-way ANOVA, followed by a post hoc Dunnett´s test ($\alpha < 0.05$).

For the comparison of the observed data and the predicted toxicity (probabilities of effect), the Independent Action (IA) model was used taking into account the single exposure results of each pesticide (Jonker et al., 2005). Under this conceptual model the pesticides in the mixture behave independently, thus the organism´s response to one of the pesticides would be the same even if the second pesticide is present or absent from the mixture, thus the toxicity of the compounds is predicted on probability statistics (Lydy et al., 2004). For quantal responses where values vary between 0 and 1 (i.e. escape response of the earthworms), the unaffected proportion can be expressed by the probabilities of nonresponse to the toxicants (Martin et al., 2009). For continuous data sets (i.e. shoot length and fresh weight of *B. rapa*) the probabilities of nonresponse (unaffected proportion) must be multiplied by the maximum value, which is the control, in order to obtain the mixture toxicity suggested by the IA model (Martin et al., 2009).

5.3 Results and Discussion

5.3.1. Chemical analysis

The results showed that pesticide concentration is in general higher in the 0-10 cm layer than in the 10-20 cm (Table 5.1). This was expected since the application of the pesticide was made at the soil surface, and during the experiment the addition of water could have contributed to a further dissipation of the pesticide along the test period.

Table 5.1: Chemical analysis of pesticide residues in soil collected in the upper layer (0-10 cm) and lower layer (10-20 cm) of the STEM after 28 d of exposure. *S* is for spirodiclofen and *D* is for dimethoate. ND is for not detected. All units are in mg a.i./Kg soil.

Nominal concentration (mg a.i./Kg)	**Spirodiclofen (0 - 10 cm)**	**Spirodiclofen (10 - 20 cm)**	**Dimethoate (0 – 10 cm)**	**Dimethoate (10 – 20 cm)**
0.06 S (Field dose)	0.049	0.001	-	-
0.3 S (5*field dose)	0.029	0.004	-	-
0.6 S (10* field dose)	0.042	0.002	-	-
0.3 D (Field dose)	-	-	0.112	ND
1.5 D (5* field dose)	-	-	0.013	ND
3 D (10* field dose)	-	-	0.025	0.001
Mix 1 (0.06 S + 0.3 D)	0.019	0.025	0.028	ND
Mix 5 (0.3 S + 1.5 D)	0.003	ND	0.008	0.009
Mix 10 (0.6 S + 1.5 D)	0.001	0.002	0.048	ND

Both pesticides are considered non-persistent in soil, which is corroborated by their soil degradation (days), since dimethoate has a DT_{90} (laboratory at 20º C) of 10.2 days and spirodiclofen a DT_{90} (laboratory at 20 ºC) of 24 days (http://sitem.herts.ac.uk/aeru/footprint/en/index.htm). Furthermore, both chemicals have a low leaching potential (dimethoate = 1.05 and spirodiclofen = -0.42), which indicates their low leachability (http://sitem.herts.ac.uk/aeru/footprint/en/index.htm). These parameters regarding the environmental fate of dimethoate and spirodiclofen should explain the reason why just a small amount of pesticide residues were detected after the 28 days of exposure, even in the highest concentrations applied to the soil.

Comparing the analyses performed with soil spiked with dimethoate field dose with the applied at 5 and 10 times the field dose, only a small amount of pesticide was retrieved in the soil samples of the upper layer (0-10 cm). In addition, after the 28 days of exposure, at the lower layer of 10-20 cm, a small amount of pesticide residues was identified only in the highest concentration tested. The same occurred in all the binary mixtures performed, where just a residual amount of dimethoate was found in the lower layer. A similar pattern

in terms of pesticide recovery was observed for spirodiclofen, since in the lower layer just a few amount of this acaricide was obtained in the analysis performed, even in the highest applications made in both single and binary mixtures.

5.3.2 *Brassica rapa*

There were no statistical differences in the shoot length and fresh weight (FW) after exposure to the pesticides dimethoate and spirodiclofen in any of the treatments performed (Fig. 5.5). However, a decrease of about 50% in plants length and FW was observed at the higher concentrations of dimethoate as well as in the concentration of 0.3 mg spirodiclofen/Kg soil. In the binary experiment no evident effects in both endpoints were found, but with increasing dosages of pesticides a trend in decreasing FW and length could be detected (Fig. 5.5).

The field dose (FD) and thus the ecological relevant concentration did not cause any impairment in the growth ability of this plant species. Indeed the small augment in the FW at the recommended dose of spirodiclofen and the increase in both endpoints at the field dose of dimethoate may indicate that these two pesticides did not affect the growth ability of *B. rapa* at recommended doses (Fig. 5.5). Previous works using the recommended dose of dimethoate in field applications did not observe any detrimental effects in the growth pattern of several *Brassica* species (Sarwar et al., 2003, Rana et al., 2007). Recent studies where spirodiclofen was applied, at recommended doses, did not observe any impact in several plant species (Fountain et al., 2010, Hu et al., 2010, Van Leeuwen et al., 2010).

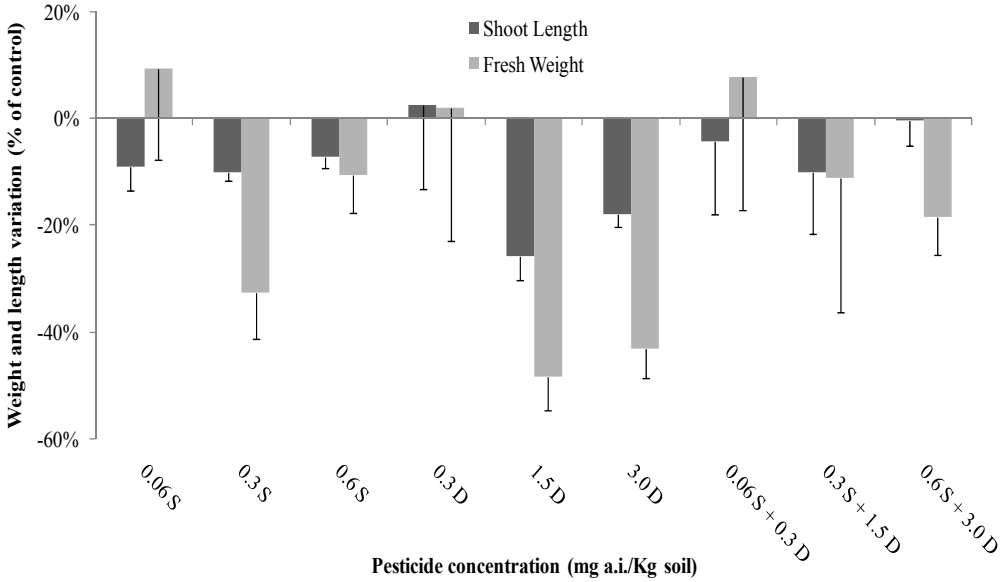

Fig. 5.5 – Fresh weight (mg/plant) and shoot length (cm/plant) variation of *Brassica rapa* after the application of dimethoate, spirodiclofen and a binary mixture of the two PPPs in the STEM for 28 d. All units (nominal values) are in mg a.i./Kg soil. *D* indicates dimethoate; *S* indicates spirodiclofen.

The decrease in both length and FW after application of spirodiclofen and dimethoate at 5 and 10 times the FD was more substantial than when the two pesticides were applied at these respective concentrations in the binary mixture. For instance, the length decreased 10 and 26% after exposure to 5 times the field dose of spirodiclofen and dimethoate respectively, but when the two pesticides were applied together the reduction was around 10% (Fig. 5.5). This demonstrated that when combined the pesticide did not cause an effect as strong as expected considering their effects when tested in the single exposures. The same occurred in the FW of the plants, since in the single exposures a reduction in 33% was observed after exposure to spirodiclofen and a reduction in 49% was detected after exposure to dimethoate (Fig. 5.5). Nevertheless, in the joint experiment the reduction in the FW was only of 11%, thus when the two pesticides were applied simultaneously their effect was not as steep as predicted when considering their single exposure effects.

Regarding the concentrations of 10 times the field dose, the decrease in plants' length was 7 and 18% after exposure to spirodiclofen and dimethoate, respectively, but in the mixture the reduction in plants growth was only 0.4% in comparison with the control (Fig. 5.5). Concerning the FW variation, after exposure to spirodiclofen plants loss was of 11% and after dimethoate exposure it was around 43%; however, in the binary mixture the reduction in FW was 18%. Again, the same pattern was observed with the effects predicted from the single exposures conducted simultaneously being higher than in the observed combination of the two pesticides.

An overlook at the data from the plant shoot length and fresh weight indicates that antagonism occurred in the binary mixtures made with these two pesticides. This was further corroborated when the mixture toxicity suggested by the IA model was compared with the actually observed data from the mixtures (Table 5.2). In the binary mixture made with the field dose the difference between observed and predicted mixture toxicity was not that steep, since the length observed was 9.8 cm and the prediction made by the IA was 9.4 cm, thus the prediction fell in the standard deviation interval of the observed result (Table 5.2). In the combinations made with 5 times the field dose, the observed length was 9.5 cm and the predicted result was 6.9 cm, therefore it should be expected, according to the IA model based on the single exposure effects, that the plants should have grown just 6.9 cm when they actually reached 9.5 cm (Table 5.2). Since the mixture effect was not as strong as expected, an interaction occurred between the two pesticides. The same antagonistic pattern was observed in the combination made with 10 times the field dose, where the observed value of 10.5cm was higher than the predicted 7.5 cm suggested by the IA model taking into consideration the single exposure effects.

Regarding the effects of FW, at the field dose application rate the difference from the observed value (222 mg) and the predicted value (200 mg) was almost neglectable, but in the combinations with 5 times the FD there was a discrepancy between the observed value of 191 mg and the predicted FW of 68 mg according to the IA model. The same pattern can be observed in the highest concentration of the binary mixture performed, where the observed value (177mg) was higher than the predicted IA value (100mg) (Table 5.2).

Therefore, the effects predicted by the IA model, based on the single exposure effects, were not corroborated by the observed values of the combined effect in the length and FW of *B. rapa*, and the discrepancy between the observed and predicted values seems to increase with the concentrations used in the binary mixtures. The same result (antagonistic deviation) has been obtained in a previous study made by the same authors, where the binary mixture of dimethoate and spirodiclofen was less toxic to the length and FW of *B. rapa* grown for a period of 21 days in LUFA soil than it was expected by the single exposures (Santos *et al*., submitted).

Table 5.2: Observed values (mean net response and standard error) and predicted mixture toxicity values calculated by the independent action model using the probabilities of nonresponse (unaffected proportion of the endpoint measured). The endpoint measured for the earthworm *Eisenia andrei* was the proportion of worms in the soil lower layer (10-20 cm) and for *Brassica rapa* were the shoot length (in cm/plant) and fresh weight (in mg/plant). *S* is for spirodiclofen and *D* is for dimethoate. All units of the treatments (nominal concentrations) are in mg a.i./Kg soil.

Treatment	***Eisenia andrei*** **(proportion 10-20cm)**	***Brassica rapa*** **(shoot length)**	***Brassica rapa*** **(fresh weight)**
Control	0.12 (0.06 – 0.22)	10.7 (10.3 – 11.1)	235 (228-242)
0.06 S	0.53 (0.33 – 0.74)	9.4 (8.7 - 10.0)	215 (152 – 277)
0.3 D	0.73 (0.68 - 0.79)	10.8 (7.9 – 13.8)	219 (125 – 313)
Mix 1 (0.06 S + 0.3D)	0.66 (0.51 – 0.82)	9.8 (7.6 – 12.1)	222 (126 – 318)
Predicted Mix 1	0.88	9.4	200
0.3 S	0.27 (0.05 – 0.57)	9.5 (9.2 – 9.7)	143 (122 – 165)
1.5 D	0.53 (0.32 – 0.74)	7.8 (7.2 – 8.4)	112 (100 – 123)
Mix 5 (0.3 S + 1.5 D)	0.30 (0.20 – 0.41)	9.5 (7.6 – 11.4)	191 (108 – 273)
Predicted Mix 5	0.66	6.9	68
0.6 S	0.57 (0.45 – 0.68)	9.8 (9.4 – 10.3)	192 (168 – 215)
3 D	0.40 (0.23 – 0.57)	8.7 (8.3 – 9.0)	123 (111 – 134)
Mix 10 (0.6 S + 3 D)	0.83 (0.78 – 0.89)	10.5 (9.7 – 11.4)	177 (155 – 200)
Predicted Mix 10	0.74	7.9	100

5.3.3 *Eisenia andrei*

The exposure of earthworms to the two pesticides caused a decrease in their weight, with the exception of the exposure to 0.3 mg a.i. spirodiclofen /Kg soil where a residual increase in the biomass (0.4%) was observed (Fig. 5.6). With the exception of this spirodiclofen concentration, there was a general decrease consistent with increasing concentrations of spirodiclofen and dimethoate, and also with increasing concentrations of both pesticides in the binary mixtures performed. The loss of weight during the experimental period, even in control conditions, has been previously reported in several tests performed with terrestrial earthworms concerning the application of dimethoate (Dalby et al., 1995) as well as other pesticides, in both laboratory and field studies (Van Gestel and Dis, 1988; Liang and Zhou, 2003; Reinecke and Reinecke, 2007).

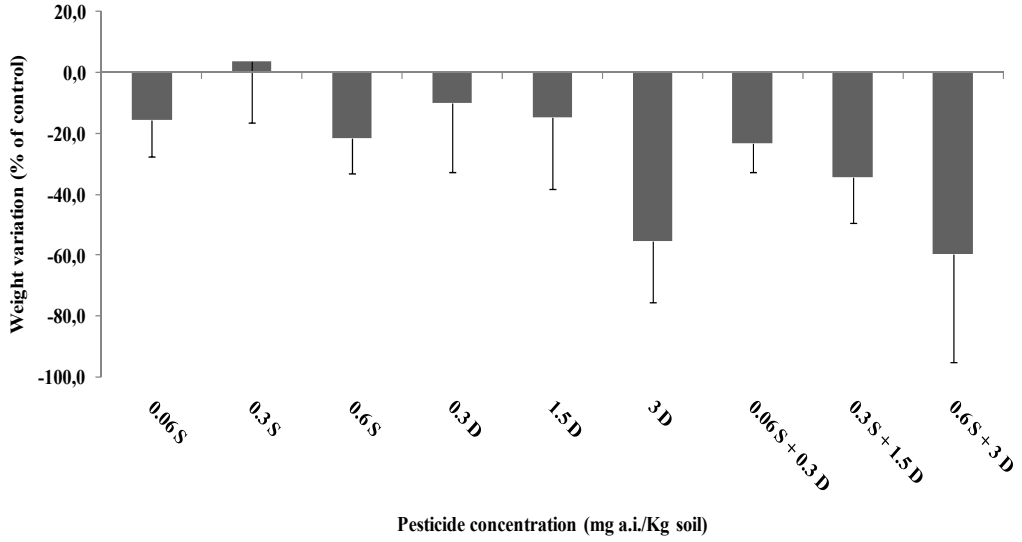

Fig. 5.6 – Weight variation (as % of control) with standard error of *Eisenia andrei* after the application of dimethoate (D), spirodiclofen (S) and a binary mixture of the two PPPs in the STEM for 28 d. All units (nominal values) are in mg a.i./Kg soil.

Regarding the distribution of earthworms (vertical distribution) in the control, more than 70% of the worms were found in the upper layer (0-10 cm) of the STEM soil column (Fig. 5.7). Finding the earthworms in the upper soil layer should be regarded as the

"normal" distribution of this epigeic species (Langdon et al., 2005), and deviations from this pattern should be interpreted as an escape response from unfavorable conditions.

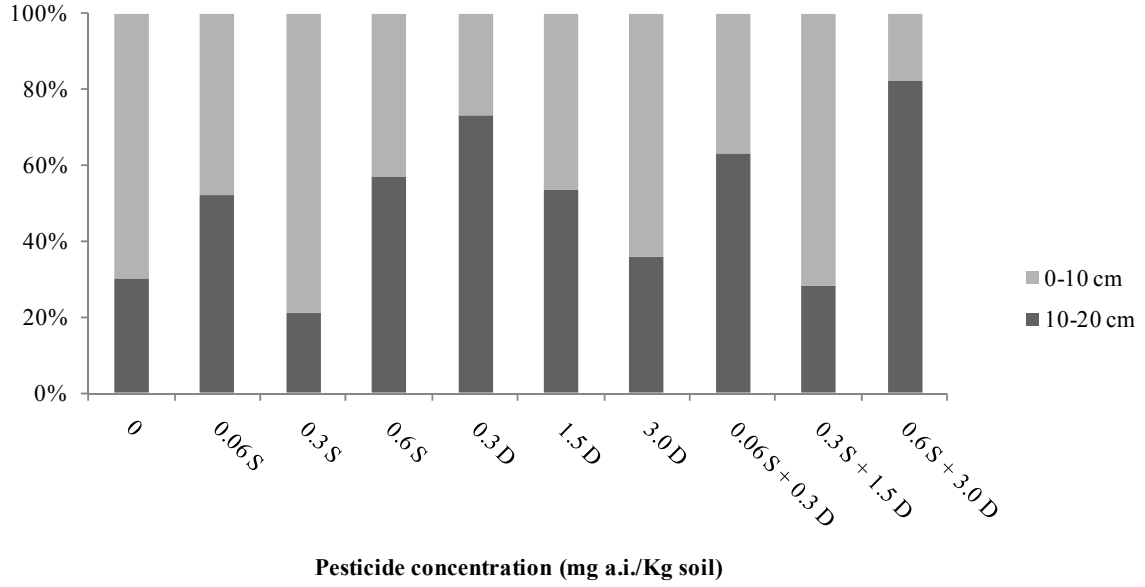

Fig. 5.7 - Proportion of *Eisenia andrei* found along the STEM soil column in 0-10 cm upper layer and 10-20 cm lower layer, after 28 days of exposure to dimethoate (D), spirodiclofen (S) and a binary mixture of the two pesticides. All units (nominal values) are in mg a.i./Kg soil.

Following the application of the acaricide spirodiclofen, more worms were found in the lower layer of 10-20 cm layer at the field dose and 10 times the field dose than in the control conditions, although in the concentration with 5 times the field dose 79% of the worms were in the upper layer (Fig. 5.7). There was not a dose response trend in the escape response of the earthworms with increasing doses of this pesticide, but a correlation between the pesticide concentrations detected in the upper layer (Table 5.1) and the number of earthworms recovered seems to exist: at higher concentrations of this acaricide in the soil fewer worms were observed. This result has been observed for spirodiclofen with other test-species following its application in soil (Santos et al., 2010).

At the field dose of dimethoate more earthworms were found in the lower layer (73%), but with increasing dosages of this insecticide the number of individuals found in the upper layer increased regularly (Fig. 5.7). This apparent contradictory result should be interpreted taking in consideration the mode of action of dimethoate in earthworms. Organophosphate pesticides are known to cause a disruption in acetylcholinesterase inhibiting its enzymatic activity in earthworms (Rao et al., 2003), which could lead to an impairment in the regular behaviour of these organisms.

Some constrictions in earthworm's body were observed in the highest concentration of dimethoate and also in the binary combination with 10 times the field dose for the two pesticides. These constrictions were associated with a more languid behaviour observed when compared to the animals presented in control conditions, or even in earthworms exposed to lower concentrations of the pesticides. When the affected worms were poked they did not respond immediately to the contact, revealing a lack of response to the stimuli, a behaviour which has been observed also in other studies dealing with similar organophosphate pesticides (Booth et al., 2000; Rao et al., 2003; Novais et al., 2010). Muscle contractions are coordinated by nervous impulses, which are mediated by AChE activity (Stenersen, 1979), thus the inhibition of this enzyme could explain the languid behaviour and concomitant incapacity of escaping from the more contaminated portion of the soil.

The percentage of worms observed in the lower layer at the field dose of the binary mixture was 66 % (Table 5.2), although according to the suggestion made by the IA model, based on the single exposure responses, around 88% of the worms should have migrated to the 10-20 cm portion of the soil (Table 5.2). At 5 times the field dose, 30 % of the worms were in the lower layer, and the prediction made by IA was that more than the double amount of worms should have escaped (66 %). Thus in these two concentrations the number of worms in the less contaminated area of the soil was less than it should be expected by the escape response in the single exposures. It can be said that antagonism occurred at these concentrations in the mixtures, since the escape response predicted by the reference model was above the observed results, therefore the effect was not as strong as predicted by the model (Table 5.2). This antagonistic deviation was also observed in a

previous study using the same binary mixture, where the avoidance behaviour of the terrestrial isopod *Porcellionides pruinosus* was evaluated (Santos et al., 2010).

In the highest mixture performed the observed value of 83 % of worms in the lower layer was higher than the predicted escape of 74 % obtained by the IA model (Table 5.2). These results might mean that a synergistic pattern took place as the response observed was higher than the predicted by the individual responses. This should be linked with the observed response escape in the single exposures made with the highest concentrations of the two pesticides. After exposure to the highest concentration of dimethoate in the single exposure, most earthworms stayed in the upper layer, but the majority escaped to the lower layer after exposure to the highest dose of spirodiclofen. When these pesticides were jointly applied, the worms escaped more than expected, and this could be due to the presence of spirodiclofen in the mixture. This acaricide could be the responsible for sending the "aware signal" to the earthworms, which had the opportunity to escape and avoid being entrapped in the more contaminated section as they were in the single exposure to the insecticide dimethoate.

5.4 Conclusions

At the field dose (ecological relevant concentration) no effects were observed in all the endpoints measures in both *B. rapa* and *E. andrei*. The binary mixtures showed an antagonistic effect on shoot length and fresh weight of *B. rapa* in all the concentrations tested. Although there was an increase in earthworms weight loss in comparison with the control in all microcosms treated with pesticides, the differences were not statistically significant. Nevertheless, in the binary mixture performed with 10 times the field dose, more worms have escaped than it was predicted by the reference model, indicating synergism when both pesticides were applied jointly at the highest concentration. In this way, behaviour escape response seems to be an important and sensitive evaluating parameter for these pesticides. The migration to the lower layers may have an ecological effect in the edaphic ecosystems; hence the earthworms could be impaired of regulating the turn-over of organic matter on soil surface with severe consequences to edaphic

equilibrium. The small-scale terrestrial ecosystem (STEM) presented in this study had a good potential as a predictive tool to assess environmental pollutants effects of combined pesticides to non-target organisms.

References

Alonso, E., Nunez, M.G., Carbonell, G., Fernández, C., Tarazona, J.V., 2009. Bioaccumulation assessment via an adapted multi-species soil system (MS.3) and its application using cadmium. Ecotox. Environ. Safe. 72, 1038-1044.

Booth, L.H., Hodge, S., O´Halloran, K., 2000. Use of cholinesterase in *Aporrectodea caliginosa* (Oligochaeta: Lumbricidae) to detect organophosphate contamination: comparison of laboratory test, mesocosms, and field studies. Environ. Toxicol. Chem. 19, 417-422.

Burrows, L.A., Edwards, C.A., 2002. The use of integrated soil microcosms to predict effects of pesticides on soil ecosystems. Eur. J. Soil Biol. 38, 245-249.

Cassee, F.R., Sühnel, J., Groten, J.P., Feron, V.J., 1999. Toxicology of chemical mixtures, in: Ballantyne, B., Marrs, T., Syversen, T. (Eds.), General and Applied Toxicology, Macmillan Reference Ltd., London, pp. 303–319.

Dalby, P.R., Baker, G.H., Smith, S.E., 1995. Glyphosate, 2,4, DB and dimethoate: effects on earthworms survival and growth. Soil Biol. Biochem. 27, 1661-1662.

De Laender, F., De Schamphelaere, K.A.C., Vanrolleghem, P.A., Janssen, C.R., 2009. Comparing ecotoxicological effect concentrations of chemicals established in multi-species vs. single-species toxicity test systems. Ecotox. Environ. Safe. 78, 310-315.

Edwards, C.A., 2002. Assessing the effects of environmental pollutants on soil organisms, communities, processes and ecosystems. Eur. J. Soil Biol. 38, 225-231.

Fountain, M.T., Harris, A.L., Cross, J.V., 2010. The use of surfactants to enhance acaricide control of *Phytonemus pallidus* (Acari: Tarsonemidae) in strawberry. Crop Prot. 29 , 1286-1292.

Gange, A.C., Brown, V.K., Farmer, L.M., 1992. Effects of pesticides on the germination of weed seeds: implications for manipulated experiments. J. Appl. Ecol. 29, 3030-310**.**

http://sitem.herts.ac.uk/aeru/footprint/en/index.htm

Hu, J., Wang, C., Wang, J., You, Y., Chen, F., 2010. Monitoring of resistance to spirodiclofen and five other acaricides in *Panonychus citri* collected from Chinese citrus orchards. Pest Manag. Sci. 66, 1025-1030.

Jong, F.M.W., Snoob, G.R., van de Zande, J.C., 2008. Estimated nationwide effects of pesticide spray drift on terrestrial habitats in the Netherlands. J. Environ. Manage. 86, 721–730.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose–response analysis. Environ. Toxicol. Chem. 24, 2701–2713.

Langdon, C.J., Hoson, M.E:, Arnold, R.E., Black, S., 2005. Survival, Pb-uptake and behaviour of three species of earthworms in Pb treated soils determined using and OECE-style toxicity test and a soil avoidance test. Environ. Pollut. 138, 368-375.

Liang, J., Zhou, Q., 2003. Single and binary-combined toxicity of methamidophos, acetochlor and copper acting on earthworms *Esisenia foelide*. Bull. Environ. Contam. Toxicol. 71, 1158–1166.

Lemos, M.F.L., van Gestel, C.A.M., Soares, A.M.V.M., 2010. Developmental toxicity of endocrine disrupters bisphenol A and vinclozolin in a terrestrial isopod. Arch. Environ. Contam. Toxicol. 59, 274-281.

Loureiro, S., Amorim, M., Campos, B., Rodrigues, S., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchystraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157, 625-623.

Loureiro, S., Svendsen, C., Ferreria, A.L.G., Pinheiro, C., Ribeiro, F., Soares, A.M.V.M., 2010. Toxicity of three binary mixtures to *Daphnia magna*: comparing chemical modes of action and deviations from conceptual models. Environ. Toxicol. Chem. 29, 1716-1726.

Lydy M.J. & Linck S.L, 2003. Assessing the impact of triazine herbicides on organophosphate insecticide toxicity to the earthworm *Eisenia fetida*. Arch. Environ. Contam. Toxicol. 45, 343-349.

Martikainen, E., 1996. Toxicity of dimethoate to some soil animal species in different soil types. Ecotox. Environ. Safe. 33, 128–136.

Martin, H.L., Svendsen, C., Lister, L.J., Gomez-Eyles, J.L., Spurgeon, D.J., 2009. Measurement and modeling of the toxicity of binary mixtures in the nematode *Caenorhabidtis elegans* - a test for independent action. Environ. Toxicol. Chem. 28, 97-104.

Novais, S.C., Soares, A.M.V.M., Amorim; M.J.B., 2010. Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticide testing? Chemosphere 79, 233-237.

Rana, Z.A., Shazhad, M.A., Malik, N.A., Saleem, A., 2007. Efficacy of different insecticides and DC-Plus against mustard aphid, *Lipaphis erysimi* (Kalt), J. Agri. Res. 45, 221-224.

Rao, J. V.,Pavan, Y.S., MAdhavendra, S.S., 2003. Toxic effects of chlorpyrifos on morphology and acetylcholinesterase activity in the earthworms *Eisenia foetida*. Ecotox. Environ. Safe. 54, 296-301.

Reinecke S.A. and Reinecke, A.J., 2007. Biomarker response and biomass change of earthworms exposed to chlorpyrifos in microcosms. Ecotox. Environ. Safe. 66, 92-101.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2010. Joint effects of three plant protection products to the isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. Chemosphere 80 , 1021-1030.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., (submitted). Joint effects of three plant protection products to the higher plants *Triticum aestivum* (L.) and *Brassica rapa* (L.).

Sarwar, M., Ahmad, N., Siddiqui, Q.H., Rajput, A.A., 2003. Efficienccy of different chemicals on canola strain rainbow (*Brassica napus* L.) for aphids control. Asian J. Plant Sci. 2, 831-833.

Stenersen, J., 1979. Action of pesticides on earthworms. Part I. The toxicity of cholinesterase inhibiting insecticides to earthworms as evaluated by laboratory testing. Pest. Sci. 10, 66-74.

Van Gestel, Dis, W.A., 1988. The influence of soil characteristics on the toxicity of four chemicals to the earthworm Eisenia fetida andrei (Oligochaeta). Biol. Fertil. Soils 6, 262-265.

Van Leeuwen, T.V., Vontas, J., Tsagkasakou, A:, Dermauw, W., Tirry, L., 2010. Acaricide resistance mechanism in the two-spotted mite Tetranychus urticae and other important Acari: a review. Insect Biochem. Mol. Biol. 40, 563-572.

Vieira, M.M., 2009. Vendas de produtos fitofarmacêuticos em Portugal em 2008. Direcção Geral de Agricultura e Desenvolvimento Rural. Ministério da Agricultura e do Desenvolvimento Rural, Portugal.

Wolf, C., Schnorbach, H.-J., 2002. Ecobiological profile of the acaricide spirodiclofen. Bayer Crop. Sci J. 55, 177-906.

6. EVALUATION OF THE JOINT EFFECT OF GLYPHOSATE AND DIMETHOATE USING A SMALL-SCALE TERRESTRIAL ECOSYSTEM

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Abstract

In the present work a small-scale terrestrial ecosystem (STEM) containing a Mediterranean agricultural soil was used to survey the effects of the combination of the herbicide glyphosate and the insecticide dimethoate, mimicking real scenarios of exposure. Earthworms (*Eisenia andrei*), isopods (*Porcellionides pruinosus),* turnip seeds (*Brassica rapa*) and bait-lamina strips were placed in the STEM. The results showed that the application of the recommended field dose (ecological relevant concentration) of both pesticides did not cause any effect in the growing ability of earthworms and plants. The application of the herbicide, even at 5 and 10 times the field dose, increased feeding activity in soil (bait-lamina test), although the application of dimethoate led to a decrease in feeding activity in all concentrations tested. The binary mixtures performed showed that according to the independent action model, synergism (higher effect than expected from the single exposures) was observed in both the shoot length and fresh weight of B. *rapa* at 5 times the field dose, but antagonism was observed at 10 times the field dose. Regarding the germination success, antagonism was observed at the field dose, but synergism occurred in the mixtures made with 5 and 10 times the field dose. There was a decrease in the earthworm's weight in all concentrations tested, although no statistical differences were observed in any of the treatments made. Regarding the vertical distribution of *E. andrei* in all the concentrations of the two pesticides, antagonism was observed (worms escaped less than should be expected from the single exposures made) in all concentrations. In all the binary mixtures performed a decrease in the feeding activity (bait-lamina consumption) of the soil fauna was observed, indicating synergism in all three binary mixtures. From the four biomarkers assessed in isopods (Catalase, AChE, GST, and LPO), only a significant decrease in the AChE activity upon dimethoate and the binary mixtures exposures performed with the field dose was observed and on LPO at the field doses of single and binary exposures.

6.1 Introduction

Microcosms have been proposed as an optional tool to evaluate the effects of chemicals in both structural and functional endpoints in soil ecosystems in order to achieve a greater realism in ecotoxicological evaluation of chemicals to non-target organisms (Edwards et al., 2002). The effects of several classes of pollutants have been tested in microcosm design experiments, using various test compartments and methodology in terms of numbers of species introduced, time period of evaluation and soil type used as exposure medium (Lukkari et al., 2006). The option for an integrated microcosm experiment should try to establish a possible link between what happens in single species exposure tests made in laboratory conditions and the effects expected in field conditions (Løkke and Van Gestel, 1998).

The application of plant protection products (PPP) in agricultural fields to control pests and achieve maximum harvest yield can pose threats to non-target organisms present in soil ecosystems (Giller et al., 1997). Since the majority of crops need more than just one type of PPP application during its growing cycle, this could pose an extra problem in terms of the action of combined agents to soil populations living in the vicinity of these exposure sites (Santos et al., 2010a). Consequently, it seems important to study the effects of combined PPP to non-target organisms, such as plants, earthworms and other faunal communities present in soil ecosystems (Matsumura, 1987).

The Independent Action (IA) model has been established as a reference model for the assessment of pesticide mixtures of chemicals with different modes of action (Svendsen et al., 2010). Theoretically this model has subjacent the principle that the probability of the effect of one chemical is independent from the probability of the effect of the other chemical present in the mixture (Loureiro *et al.*, 2010). However, in some mixtures, interactions between the components can occur, resulting in antagonism, when the mixture toxicity is lower than expected from the toxicities of single components, or synergism whereas the joint effects are stronger than it should be expected by the individual components toxicity (Jonker et al., 2005).

Earthworms are responsible for the redistribution of organic material in soil, increasing soil penetrability and influence the supply of nutrients in soil ecosystems, thus

contributing to the augment of soil fertility (Syers and Springett, 1984). Their importance in maintaining soil structure and function is well recognized and any impact in its population dynamics should be addressed in terms of lack of services provided to soil equilibrium (Mäder et al., 2002). Non-target plants can enter in contact with pesticide residues due to spray drift from agricultural fields (Marrs et al., 1989), and this exposure may lead to impairment in plant communities (Snoo and Wit, 1998). Terrestrial isopods are important litter macrodecomposers on the soil surface that assume a key role in nutrient recycling of the plant material in edaphic ecosystems, and the impact of pesticides on biochemical indicators (biomarkers) has been used to evaluate possible detrimental effects that PPP could pose to this non-target organisms (Santos et al., 2010b). Soil organic carbon, provided by dead plants and animals, is the final result of decomposition processes promoted by microbial communities present in soil (Bronick and Lal, 2005). The application of pesticides in soil could pose a threat to these soil faunal communities, which are responsible for the organic matter decomposition (Büneman et al., 2005). Therefore it seems relevant to assess the impact of pesticides in the structure of edaphic communities, but also in their function, in terms of organic matter decomposition, and possible impairment of these ecosystems.

Two PPP with different modes of action were chosen in this work: the herbicide glyphosate, which acts in plants through the inhibition of 5-enolpyruvylshikimic acid 3-phosphate (EPSP) synthase (Huangfu et al., 2007) and the insecticide dimethoate which inhibits the action of acetylcholinesterase (AChE) enzyme activity in several arthropods (Loureiro et al., 2005) and also inhibits the synthesis or action of hydrolytic enzymes inside seeds (Gange *et al.*, 1992).

The objectives of this work were firstly to evaluate if a designed microcosm experiment could function as an useful tool to mimic the effects of pesticide application in agricultural fields and secondly to evaluate the combined effects of the two pesticides to the earthworm *Eisenia andrei*, the terrestrial isopod *Porcellionides pruinosus*, the turnip species *Brassica rapa* and to the bait-lamina strips, using the concept of independent action.

6.2 Materials and Methods

6.2.1 Test organisms and test soil

Earthworms of the epigeic species *Eisenia andrei* (Bouché) were bought from a commercial supplier and seeds of *Brassica rapa* (L.) rapid cycle (Carolina Biological Supply Company) were used. Adult isopods of the species *Porcellionides pruinosus* (15-25 mg wet weight) were used with no sex differentiation, although pregnant females and animals with lack of antennae were not used in the experimental procedure.

An agricultural soil was collected in the spring of 2010 (first 20 cm of soil) from the lower Mondego Valley (Portugal). The soil was brought from an agricultural field that has not been pesticide-treated in the last five years (Lemos *et al.*, 2010). Soil parameters include a pH (H_2O) = 7.48, organic matter content = 2.4 %, clay = 4.2 %, silt = 7.0%, sand = 88.7%, density (g/cm^3) = 2.4 and a water holding capacity = 70%. The soil was brought to lab, sieved (5 mm) and air dried prior to experimental procedures.

6.2.2 Test chemicals

The effects of glyphosate and dimethoate on test species were studied using three different concentrations, ranging from the field dose (FD), i.e. the recommended application rate, to 10 times the FD of the commercial formulation of glyphosate (Roundup®) and dimethoate (Agror®).

More specifically, the nominal concentrations for glyphosate ranged from 1.7 mg a.i./Kg dry soil (recommended labeled dose), 8.5 mg a.i/Kg dry soil (5 fold the recommended dose) to 17 mg a.i/Kg dry soil of glyphosate (10 fold the recommended dose); for dimethoate ranged from 0.3 mg active ingredient (a.i.)/Kg dry soil (recommended labeled dose), 1.5 mg a.i./Kg dry soil (5 fold the recommended dose) to 3 mg a.i./Kg dry soil (10 fold the recommended dose). The binary mixtures were made using the same concentrations of the two pesticides at the field dose, 5 times the field dose and 10 times the field dose in a one fixed ratio design (Fig. 6.1). For each treatment and

control, three replicates were made, in a total of 30 microcosms tested. Pesticides were applied at the surface of the soil layer using a common sprayer.

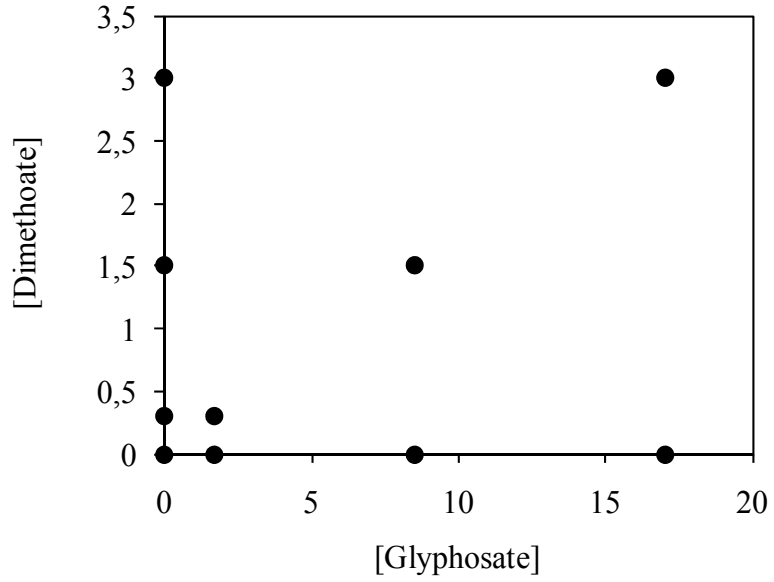

Fig. 6.1 – Experimental design of the binary combinations performed, based on the concentrations of glyphosate and dimethoate (all units are in mg a.i./Kg soil).

6.2.3 Small-scale terrestrial ecosystem (STEM)

The small-scale terrestrial ecosystems (STEM) consisted on 2mm wall cylindrical PVC pipes (12 cm diameter and 38 cm deep) with a surface area of 0.095 m^2, filled to within 5 cm of the top with agricultural soil (Fig. 6.2). Each STEM was filled with approximately 4 kg of soil. The bottom of the microcosms was covered with fine plastic gauze (1.0 mm aperture) to avoid earthworms' escaping from the microcosms (Fig. 6.2).

The STEM were placed in an acclimatized moveable cart (83 cm length * 55 cm width * 55 cm depth) with the lid hollowed in order to enclose 5 STEM. These acclimatized chambers allowed to maintain soil temperature (12 º C) and moisture of the STEM in controlled conditions. Six acclimatized chambers, each one with 5 STEM, were used in the experimental procedure.

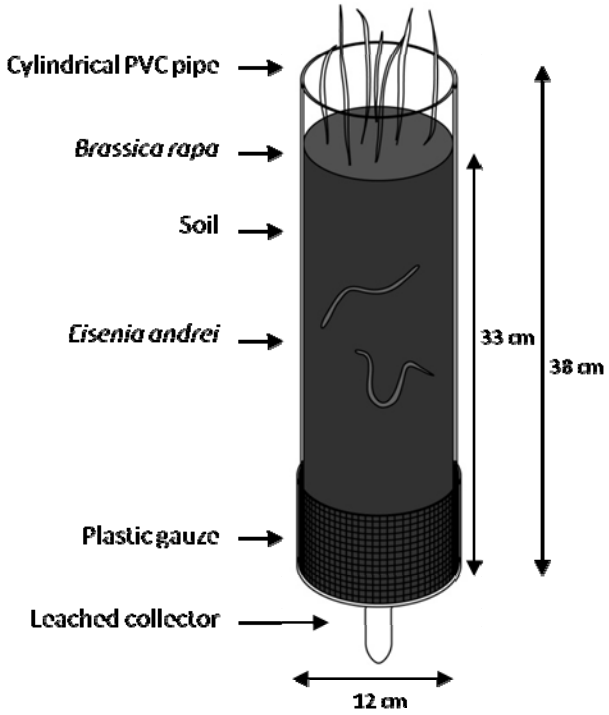

Fig. 6.2 – Small-scale terrestrial ecosystem (STEM) used in the experimental procedure.

6.2.4 Experimental Setup in STEM

Each small-scale terrestrial ecosystem (STEM) was incubated in an experimental room at 20 ± 2º C, with a 16/8 h light dark regime for a period of 28 days. Soil water content was adjusted to 70% of maximum water holding capacity (WHC), making it comparable to field moisture, and was subsequently maintained throughout the experiment by adding sterile water in order to simulate rainfall in spring normal conditions (≈ 84 mm per day). Ten adult clitellate earthworms and ten turnip seeds were introduced on soil surface of each microcosm. Earthworm weight was recorded before their introduction into the microcosms. Five isopods, and alder leaves as food source, were added to each STEM at the beginning of the test. Three bait-lamina strips were also enclosed in the soil of each STEM. After 7 d of exposure the baits were removed from the soil and the number of empty holes (counted as eaten) was recorded. At the end of 28 d exposure period the fresh weight and shoot length of *B. rapa* were measured as well as the fresh weight and depth distribution of *E. andrei* along the microcosms. After 28 d of exposure the number of

surviving *P. pruinosus* in the STEM was recorded (retrieval rate) and the animals were kept at -80º C until the enzymatic analysis were performed

6.2.5 Isopods – Biomarker evaluation

Isopods were divided in two sections: head and body. The head was used for the AChE assay and the remaining body was used for GST, CAT and LPO assays. Homogenisation of the animals was made using a sonicator (KIKA Labortechnik U2005 Control™), for approximately 5 s, using 100% amplitude, with one pulse.

6.2.5.1 Acetylcholinesterase

One isopod head per sample was homogenized using a sonicator in 500µl of potassium phosphate buffer (0.1M, pH 7.2), and the supernatants obtained after centrifugation of the homogenates (4 ºC, 3800g, 3 min) were removed and stored at -80ºC until enzymatic analysis. The AChE activity determination was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). In a 96 well microplate 250 µl of the reaction solution was added to 50µl of the sample and the absorbance was read at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

6.2.5.2 Glutathione *S*-Transferase

Glutathione *S*-Transferases (GST) activity was determined based on the method described by Habig and collaborators (1974). After sonication, 100µL of the post-mitochondrial supernatant PMS was mixed to 200µL of a reaction solution. The reaction

solution was a mixture of 4.95 ml K-phosphate buffer 0.1M (pH 6.5) with 900µL L-glutathione reduced (GSH) 10mM, and 150µL 1-chloro-2,4-dinitrobenzene (CDNB) 10mM and it was measured at 340 nm. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6 $x10^{-3}$ M^{-1} cm^{-1}.

6.2.5.3 Catalase

Catalase (CAT) activity was determined based on the method described by Clairborne (1985). We mixed 15 µl of PMS with 150µL H_2O_2 0.030M, and 135 µL K-Phosphate 0.05M (pH 7.0) and measured the decomposition of the substrate (H_2O_2) at 240 nm. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}. Ten replicates were used for each processing methodology for this enzymatic biomarker.

6.2.5.4 Lipid peroxidation

The lipid peroxidation (LPO) assay was based on the methods described by (Bird et al., 1984) and (Ohkawa et al., 1979) by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 150 µL homogenated tissue, 500 µL trichloroacetic acid sodium salt (TCA) 12% (w/v), 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v) and 400µL Tris-HCl 60mM with diethylenetriaminepentaacetic acid **(**DTPA) 0.1mM. The reaction mixture was then incubated at 100ºC in a water bath for 1h. After this, samples were centrifuged for 5 min. at 11500 rpm (25ºC). Samples were kept away from light, at 25ºC and immediately read at 535 nm. LPO is expressed as nmol TBARS hydrolyzed per minute per mg of wet weight, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

6.2.6. Bait-lamina method

Each bait-lamina consisted on a plastic strip with 16 holes filled with a mixture of cellulose, oat-bran, and active charcoal (70:27:3) and buried vertically in the soil of each STEM. After the exposure period of 7 d the baits are removed from the STEM and washed with tap water, the number of eaten baits (light fall through the bait) and non eaten baits (light does not fall through the baits) was recorded (Kratz, 1998).

6.2.7 Chemical analysis

Soil chemical analysis was performed after 28 days of exposure. Soil samples from the upper soil layer (0 - 10 cm) and the lower soil layer (10 - 20 cm) were taken and sent for chemical analysis at Marchwood Scientific Services, Southampton, UK. The method used for the analysis of soil samples for glyphosate and dimethoate comprised a first procedure for soil to air dry and ground; then 1-2 gram sample were extracted with acidified acetonitrile. The sample was then filtered and the filtrate subjected to further analysis by Liquid Chromatography-tandem Mass Spectrometry following a pre-treatment buffering stage. The instrument used for the analysis was an Agilent 6410 Triple quad LCMS-MS. Standards were prepared in solvents at 7 levels with recoveries ranging from 80-120%.

6.2.8 Statistical analysis

Differences in plants' fresh weight and length, earthworm's fresh weight and number of holes eaten in the bait-lamina strips in the microcosms where pesticide was applied were compared with control microcosms using a one-way ANOVA, followed by a post hoc Dunnett´s test. Differences in enzymatic activity between isopods in control and in microcosms treated with the pesticides were compared with the Kruskal-Wallis test (ANOVA on ranks). The concentration after which 50% of the animals were found dead (LC_{50}) was calculated using a logistic equation. Since the enzymatic data provided by the biomarkers evaluation were not normally distributed and data transformation did not

correct for normality, a Kruskal–Wallis ANOVA on Ranks was performed, followed by the Dunnett's method when significant differences were found.

The Independent Action (IA) model can be used for the comparison of the observed data and the predicted toxicity (probabilities of effect), taking into account the single exposure results of each pesticide (Jonker et al., 2005). According to this reference model the probability of effect of a given organism to one compound is independent from the probability of the effect of the same organism being exposed to a second compound, meaning that the resultant mixture toxicity can be predicted based on probability statistics (Köneman and Pieters, 1996). For quantal responses which values vary between 0 and 1 (i.e. escape response of the earthworms), the unaffected proportion can be expressed by the probabilities of nonresponse to the toxicants (Martin et al., 2009). For continuous data sets (i.e. vegetative vigor of *B. rapa*) the probabilities of nonresponse (unaffected proportion) must be multiplied by the maximum value, which is the control, in order to obtain the mixture toxicity suggested by the IA model (Martin et al., 2009).

6.3. Results and Discussion

6.3.1. Chemical analysis

The results showed that pesticide concentration in the 0-10 cm layer was always higher than the layer of 10-20 cm (Table 6.1). This was expected, since the application of the pesticide was made at the soil surface, and during the experiment the addition of water could have contributed to a further dissipation of the pesticide along the test period.

Glyphosate is considered moderately persistent in soil, with an half-live of between 1.1 - 13 days, and dimethoate is of low persistence in the soil environment with an half-life of 4-16 days (Wauchope et al., 1992). According to the analysis, after the 28 days of exposure, in both soil layers, only small amount of pesticide residues was retrieved (Table 6.1). The rapid dissipation observed for both glyphosate and dimethoate seems to confirm previous data concerning the half-life of these pesticides in soil.

Table 6.1: Chemical analysis of pesticide residues in soil collected in the upper layer (0-10 cm) and lower layer (10-20 cm) of the STEM after 28 d of exposure. *G* is for glyphosate and *D* is for dimethoate. ND is for not detected and FD is for field dose. All units are in mg a.i./Kg soil.

Nominal concentration (mg a.i./Kg)	**Glyphosate (0 - 10 cm)**	**Glyphosate (10 - 20 cm)**	**Dimethoate (0 – 10 cm)**	**Dimethoate (10 – 20 cm)**
1.7 G (Field dose)	0.12	< 0.05	-	-
8.5 G (5* FD)	2.13	< 0.05	-	-
17 G (10* FD)	0.91	0.25	-	-
0.3 D (Field dose)	-	-	< 10	< 10
1.5 D (5* FD)	-	-	0.02	< 10
3 D (10* FD)	-	-	0.04	< 10
Mix 1 (1.7 G + 0.3 D)	ND	< 50	0.02	< 10
Mix 5 (8.5 G + 1.5 D)	0.36	< 50	0.04	< 10
Mix 10 (17 G + 1.5 D)	2.41	< 50	0.07	< 10

6.3.2 *Brassica rapa*

Regarding the exposure to glyphosate, there were statistical differences in the fresh weight and shoot length of *B. rapa* after exposure to 5 times (8.5 mg glyphosate/Kg soil) the glyphosate field dose (Fig. 6.3). The number of plants that germinated after the 28 days of exposure (germination success) was significantly lower in the concentration made with 5 and 10 (17 mg glyphosate/Kg soil) times the field dose. After exposure to the insecticide dimethoate, no differences in length, FW or germination success were observed in any of the single exposures performed (Fig. 6.3). In the binary mixtures experiment there were effects in the length and FW of the plants at concentrations made with 5 and 10 times the field dose of both pesticides but no effects in the germination success were observed (Fig. 6.3).

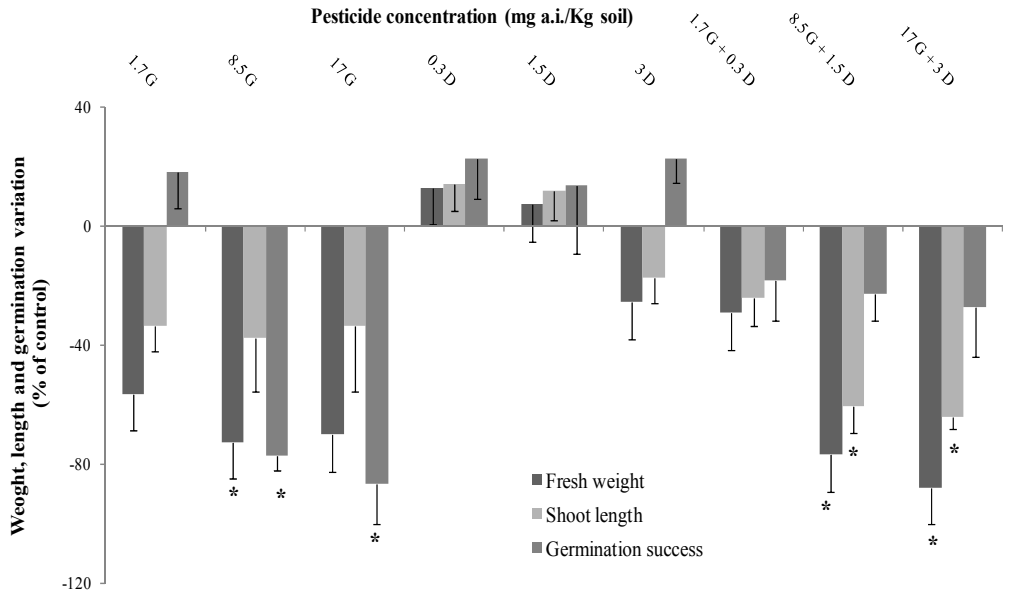

Fig. 6.3 – Variation (as percentage of control) of the fresh weight (mg/plant), shoot length (cm/ plant), and germination success (number of plants/STEM) of *Brassica rapa* after the application of glyphosate, dimethoate, and binary mixtures of the two PPPs in the STEM for 28 d. All units (nominal values) are in mg a.i./Kg soil. G indicates glyphosate; D indicates dimethoate. (* indicates statistical differences (Dunnett´s method, $p<0.05$)).

There was a decrease around 33 % in shoot length after the application of glyphosate at field dose, in 35 % after exposure to 5 times the field dose of glyphosate, and in the highest concentration of glyphosate (10 times the field dose) the reduction of the shoot length was 34 % (Fig. 6.3). After exposure to the field dose of dimethoate an increase in 14 % of the shoot length was observed in comparison with control average length value. In the exposure to 5 times the field dose of dimethoate an increase of 12 % in the shoot length was detected, but after exposure to 10 times the field dose of dimethoate a decrease in length of 17 % was observed (Fig. 6.3). In the binary mixture of the field dose of the two pesticides a reduction in 24 % of the shoot length was observed (Fig. 6.3). The binary mixture resultant from the combination of these pesticides at 5 times the field dose caused a reduction of 61 % of the length in comparison with the control, and finally, at 10 times the field dose a reduction in 64 % of the shoot length was observed (Fig. 6.3).

Regarding the effects in the fresh weight, after the exposure to the recommended dose of glyphosate a reduction in 56 % was observed (Fig. 6.3). After exposure to 5 times

the field dose of glyphosate, plants fresh weight decrease 72 %, and the exposure to 10 times the field dose of glyphosate originated a decrease in 70 % in the FW (Fig. 6.3). Exposure to the field dose of dimethoate increase of 13 % in plants FW, again an increase (7 %) was observed after exposure to 5 times the field dose of dimethoate, and only at the highest concentration of this insecticide a reduction in 25 % of the fresh weight was observed (Fig. 3). The binary mixture with the field dose of these pesticides has a decrease in 29 % of the FW, and the binary mixture performed with 10 times the field dose of both pesticides originated a total reduction of 77 % in fresh weight (Fig. 6.3). The binary mixture made with 10 times the field of the pesticides caused a reduction of 88 % (Fig. 6.3).

The total number of plants per microcosm recovered after the 28 days of exposure (germination success) was also evaluated, and the result after exposure to the field dose of glyphosate was an increase in 18 %, a decrease of 77 % after exposure to 5 times the field dose of glyphosate and in the highest concentration of glyphosate a decrease in the number of plants of 86 % was observed (Fig. 6.3). After exposure to the field dose of dimethoate an increase in 23 % of the number of plants was observed, when the plants were exposed to 5 times the field dose of dimethoate an increase of 14 % was detected and after exposure to 10 times the field dose of dimethoate, again an increase of 23 % was calculated (Fig. 6.3). The binary mixture with the field dose resulted in a decrease of 18 % in the number of plants that germinated during the exposure period (Fig. 6.3). The mixture made with 5 times the field dose of both pesticides caused a decrease of 23 % in the number of plants recovered at the end of the test. Finally, the binary mixture made with 10 times the field dose resulted in a decrease of 27 % of the number of germinated plants (Fig. 6.3).

The field dose and thus the ecological relevant concentration did not cause impairment in the vegetative vigor (length and FW) or germination success of this plant species. In fact, a small increase, in comparison with control, in the number of plants collected after the 28 days of exposure in the recommended dose of glyphosate was observed. In addition, an increase in the length, FW and plants that germinated at the field dose of dimethoate in comparison with the control average weight and length, indicate that these two pesticides, did not affect the growth ability of *B. rapa* at recommended doses. Recent studies where the leaves of seedlings of *B. rapa* with approximately 14 days were treated with glyphosate have detected an effect in reproduction (number of siliques formed

per plant) of this species at concentrations below the recommended dose, although no effect in shoot dry weight were observed (Olszyk et al., 2010). A study where glyphosate was applied to 14 days old seedlings of *Brassica napus* L., determined an EC_{50} for the shoot fresh weight of 21 g a.i./ha, after 14 days of the foliar application of the herbicide (Pestemer and Zwerger, 1999). The same has been observed for the insecticide of dimethoate, where recommended dose in field and laboratory experiments did not cause any detrimental effects in the growth pattern of several *Brassica* species (Munshi et al., 1987; Sarwar et al., 2003; Zhang et al., 2006).

Looking at the mixture toxicity data from the shoot length, after exposure to the binary mixture performed with the field dose, an average length of 8.4 cm per plant was observed, and the prediction made by the independent action (IA), based on the single exposure result to the field dose of both pesticides was a length of 8.5 cm per plant (Table 6.2). The prediction was very close to the observed data, which could be an indication that these pesticides, at the field dose application rate, behave according to the reference model of IA. The fresh weight resulting from the application of the binary mixture made with the field dose of both pesticides had a result of 136 mg, although the IA prediction was a little smaller (97 mg per plant), this value was in the range interval of the standard deviation of the result of the binary mixture (Table 6.2). The germination success observed for the binary mixture at the field dose was of 5.9 plants per microcosm, but the IA predicted value was 10.6, meaning a full germination rate in each microcosm at the beginning of the test (Table 6.2). The discrepancy between the observed value and the forecast made by the reference model seems to indicate that synergism took place in this endpoint; hence the number of plants that germinated was lower than it should be expected according to the results obtained in the single exposures performed.

Table 6.2: Observed values (mean net response and standard error) and predicted mixture toxicity values calculated by the independent action model using the probabilities of nonresponse (unaffected proportion of the endpoint measured). The endpoint measured for the earthworm *Eisenia andrei* was the proportion of worms in the soil lower layer (10-20 cm); for *Brassica rapa* were the shoot length (in cm/plant), fresh weight (in mg/plant) and the germination success (number of plants per STEM); for bait-lamina was the consumption of baits (proportion of eaten baits). *G* is for glyphosate and *D* is for dimethoate. All units of the treatments (nominal concentrations) are in mg a.i./Kg soil.

Treatment (nominal concentration)	***Eisenia andrei* (proportion in 10-20 cm)**	***Brassica rapa* (shoot length)**	***Brassica rapa* (fresh weight)**	***Brassica rapa* (germination success)**	**Bait-lamina (proportion eaten baits)**
Control	0.27 (0.16 – 0.34)	11.6 (9.7 – 11.5)	201 (176 – 227)	6.5 (5.8 – 7.1)	0.67 (0.53 – 0.77)
1.7 G	0.23 (0.12 – 0.35)	7.4 (6.3 – 8.5)	84 (74 – 94)	8.6 (6.8 – 10.4)	0.83 (0.75 – 0.90)
0.3 D	0.20 (0.01 – 0.40)	12.7 (10.7 – 14.7)	216 (132 – 300)	8.9 (6.8 – 11.1)	0.47 (0.29 – 0.64)
Mix 1 (1.7 G + 0.3 D)	0.30 (0.20 – 0.40)	8.4 (7.0 – 9.8)	136 (111 – 161)	5.9 (4.6– 7.4)	0.22 (0.17 – 0.27)
Predicted Mix 1	0.39	8.5	97	10.6	0.91
8.5 G	0.10 (0.07 – 0.27)	6.9 (4.8 – 9.1)	53 (39 – 67)	1.7 (1.5 – 1.8)	0.76 (0.55 – 0.97)
1.5 D	0.30 (0.04 – 0.56)	12.4 (10.3 – 14.5)	206 (127 – 283)	8.3 (5.0 – 11.6)	0.36 (0.22 – 0.50)
Mix 5 (8.5 G + 1.5 D)	0.10 (0.01 – 0.27)	4.4 (3.7 – 5.1)	45 (38 – 51)	5.6 (4.8 – 6.6)	0.18 (0.04 – 0.41)
Predicted Mix 5	0.37	7.7	57	1.9	0.85
17 G	0.23 (0.18 – 0.29)	2.5 (1.5 – 3.4)	19 (16 – 22)	1.0 (0.7 – 1.2)	0.74 (0.52 – 0.96)
3 D	0.37 (0.14 – 0.60)	9.2 (7.8 – 10.5)	145 (106 – 180)	8.9 (7.7 – 10.1)	0.06 (0.01 – 0.14)
Mix 10 (17 G + 3 D)	0.33 (0.28 – 0.39)	4.0 (3.7 – 5.1)	23 (22 – 23)	5.3 (3.8 – 6.8)	0.11 (0.02 – 0.30)
Predicted Mix 10	0.51	2.0	14	1.2	0.75

In the binary mixture performed with 5 times the field dose, the observed value for the shoot length was 4.4 cm per plant, but the prediction made by the IA model was that plants should have grown until 7.7 cm, meaning that the effect was higher than predicted by the model, thus synergism occurred in this mixture (Table 6.2). The effect in FW of the plants was also higher than expected from the single exposures, since the observed data of 45 mg per plant was lower than the predicted effect of 57 mg per plant, a value that was similar to the observed data, but outside the confidence interval obtained for this binary mixture (Table 6.2). Thus, regarding the two vegetative vigour endpoints, length and FW, synergism took place in the binary mixture made with 5 times the field dose of each pesticide. The germination success observed for this binary mixture was 5.6 plants per microcosm, but the predicted germination success was smaller (1.9 plants per microcosm), which is an indication that the joint effect of the two pesticides was lower than the predicted effect based on the single exposures, thus antagonism was observed for this endpoint (Table 6.2).

Therefore, for the binary mixture made with 5 times the field dose, the choice of endpoint led to different deviations from the reference model, hence length and FW effects were more affected, even than expected (synergism), but for the germination success the opposite deviation (antagonism) was observed. The length and FW are endpoints closely correlated, if the growing ability is affected the weight of the plant should also be affected, thus smaller plants have less weight and vice-versa. The germination success cannot be compared directly with the other two endpoints, since the number of seeds that germinated into full grown plants is independent of the length and weight that these plants could have. Indeed, few plants could have fully germinated after the 28 days of exposure, but their weight and length could be similar to the control values for length and FW. For instance, in the highest concentration of glyphosate only three plants were found at the end of the test, but their length and FW was not statistically different from the control (Fig. 6.2). The opposite occurred in the binary mixtures made with 5 and 10 times the field dose, where differences in length and weight were observed although no differences in germination success could be encountered (Fig. 6.2). Same conclusions have been derived from other toxicity studies, where the choice of endpoint (fresh weight and number of leaves per plant) led to different and contradictory results in terms of deviations from the mixture toxicity model according to the endpoint chosen (Cedergreen and Streibig, 2006).

After exposure to the binary mixtures made with 10 times the field dose, the observed value for the shoot length (4.0 cm per plant) was higher than the predicted values made by IA model (2.0 cm per plant), thus the joint effect was smaller than expected, or by other words antagonism took place in this binary mixture (Table 6.2). The same happened when the FW observed (23 mg per plant) was higher than the predicted value made by the model (14 mg per plant) based on the data from the single exposures (Table 6.2). The germination success observed (5.3 plants per microcosm) was also higher than the prediction made by the IA model (1.2 plants per microcosm), thus the effect of the binary mixture was smaller than expected, meaning that antagonism occurred in this mixture (Table 6.2). This antagonistic pattern has been observed in other study dealing with glyphosate and dimethoate applied jointly, where the mixture was less toxic to the length and FW of *B. rapa* grown for a period of 21 days in LUFA soil than it was expected by the single exposures to both pesticides (Santos *et al*., submitted).

6.3.3 *Eisenia andrei*

Regarding the distribution of earthworms (depth distribution) in the control, more than 73 % of the worms were found in the upper layer (0-10 cm) of the soil column (Fig. 6.4) as expected by this species ecology (Langdon et al., 2005). Following the application of the herbicide glyphosate, the distribution of the worms was similar in all the treatments (Fig. 6.4). There was not a dose response pattern in the escape response of the earthworms with increasing doses of this pesticide, thus no link between the pesticide concentrations and the number earthworms recovered was observed (Table 6.1). The absence of toxic effects at recommended doses of glyphosate-based herbicide to the earthworm *Eisenia fetida* was determined in a previous study, where the avoidance behaviour of this species was not elicited at field recommended doses of this herbicide although the locomotor activity seemed to be affected (Verrel and Van Buskirk, 2004). The same response was observed in the treatments made with the insecticide dimethoate, where the majority of worms were found in the upper layer (Fig. 6.4). This behaviour has been observed in previous microcosm's experiments, where earthworm depth distribution was not altered due to the application of the organophosphate pesticides chlorpyrifos and diazinon (Hodge et al., 2000).

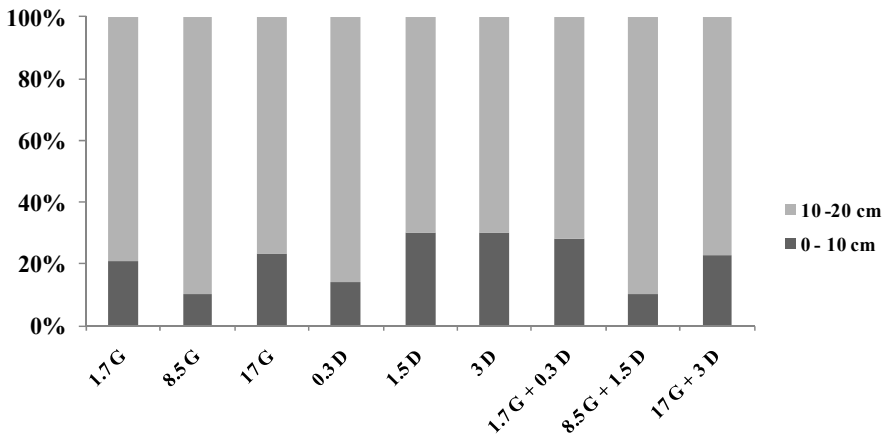

Fig. 6.4 – Proportion of *Eisenia andrei* found along the STEM soil column in 0-10 cm upper layer and 10-20 cm lower layer, after 28 days of exposure to glyphosate (G), dimethoate (D), and binary mixtures of the two pesticides. All units (nominal values) are in mg a.i./Kg soil.

In the binary mixtures increasing concentrations of both pesticides did not lead to more worms found in the lower layer of the soil column (Table 6.2). The percentage of worms in the lower layer at the field dose of the binary mixture was 30 %, and the prediction made by the IA model based on the single exposure responses was that 39 % of the worms should have migrated to the 10-20 cm portion of the soil (Table 6.2). At 5 times the field dose, 10 % of the worms were in the lower layer, and the prediction made by IA was that more than the double amount of worms should have escaped (37 %) to the lower layer (Table 6.2). The same pattern was observed in the mixture made with 10 times the field dose, where 33 % of the worms were found in the lower layer, but the prediction of the number of worms in this layer was higher (51 %) according to the IA prediction (Table 6.2). In these binary mixtures, the number of worms in the less contaminated portion of soil was lower than it should be expected by the number of worms observed in the lower layer as a result of single exposures to the pesticide. Thus, antagonism was the deviation observed in all binary mixtures regarding worm's distribution, since the effect was lower than expected by the single exposure results. This antagonistic deviation was also observed in a previous study using the same binary mixture where the avoidance behaviour of the terrestrial isopod *Porcellionides pruinosus* was evaluated (Santos et al., 2010b).

There was a decrease in the earthworm's weight in all concentrations tested, although no statistical differences were observed in any of the treatments made (Fig. 6.5). In the highest concentration of dimethoate and in the binary mixtures made with 5 and 10 times the field dose the decrease in weight was more pronounced, which seems to indicate a relation between the loss of weight and the increase in amount of pesticide in the STEM. The absence of effects in earthworm's growth after a single application of recommended doses of glyphosate and dimethoate has been observed in previous studies (Dalby et al., 1995). Thus, the field dose seems to not cause a detrimental effect in earthworms growing ability, at least in the exposure period evaluated.

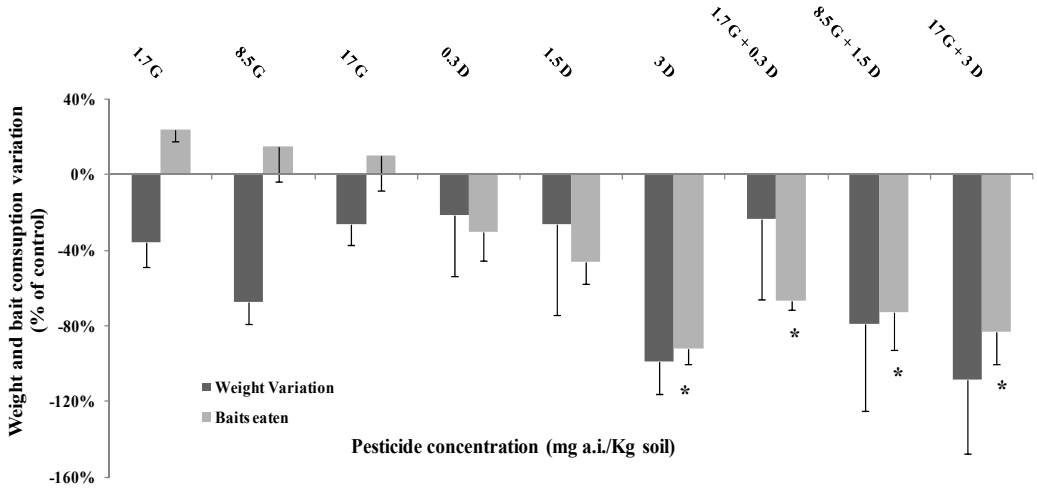

Fig. 6.5 – Variation (as percentage of control) on the fresh weight of *Eisenia andrei* and number of bait-lamina eaten after the application of glyphosate (G), dimethoate (D), and binary mixtures of the two PPPs in the STEM for 28 d. All units (nominal values) are in mg a.i./Kg soil. (* indicates statistical differences (Dunnett´s method, $p<0.05$)).

6.3.4. Bait-lamina strips

The bait-lamina test was developed to assess the feeding activity of soil organisms in different ecosystems (Kratz, 1998). After exposure to the herbicide glyphosate at a field dose concentration, the number of empty holes (counted as eaten) was higher than in the

control, and the same happened after exposure to 5 and 10 times the field dose of this herbicide (Fig. 6.5). Thus, glyphosate seemed to have triggered the feeding activity of the soil organisms in the microcosm. The same response was observed in an agricultural field treated with glyphosate where feeding activity (bait-lamina test) increased after exposure to this herbicide (Reinecke et al., 2002). The feeding activity decreased at the field dose of dimethoate and after exposure to 5 and 10 times the field dose, with statistical difference in the highest concentration of dimethoate (Fig. 6.5); the application of recommended doses of the organophosphorous pesticide chlorpyrifos in a field experiment also conveyed a significant lower consumption of the baits buried in the treated plots after a period of 50 days of exposure (Casablé et al., 2007).

In the joint toxicity evaluation, a statistically significant decrease in bait-lamina consumption was observed in all the three binary mixtures performed in comparison with the control (Fig. 6.5). The proportion of baits eaten in the binary mixture made with the field dose of both pesticides (22 %) was much smaller than the predicted consumption rate (91 %) made by the independent action model (Table 6.2). Thus, at the field dose level, results indicated that synergism (effect higher than predicted by the action of the two pesticides in the single exposure) took place in the mixture. After the exposure to 5 times the field dose the proportion of eaten baits was 18 %, but according to the IA model the percentage of empty holes should have been much higher (85 %), hence again synergism occurred between the two pesticides in this mixture (Table 6.2). After the exposure to the highest concentration of the two pesticides, the percentage of empty holes (11 %) was, once more, much smaller than the predicted effect made by the IA model (75 %), thus it can be said, that regarding bait-lamina consumption the effect of all mixtures were synergistic (Table 6.2). Although glyphosate did stimulated bait-consumption, as attested in the single exposures, the inverse effect caused by the insecticide dimethoate led to an inhibitory effect stronger than should be expected. In this case, apparently, the synergism in the toxic effect of the mixture was driven by the insecticide dimethoate.

Bait-lamina consumption has been linked to the amount of soil organisms biomass, and a strong correlation between lower bait consumption and contaminated sites and has been established (Kools et al., 2009). Furthermore, a link between bait-lamina consumption and the presence of earthworms in soil has been demonstrated in previous

studies (Van Gestel et al., 2003). The decrease in *E. andrei* weight in the binary mixtures made with 5 and 10 times the field dose may be one reason for the observed decrease in the feeding activity. Thus, feeding activity of soil organisms was a good indicator of the functional equilibrium of the soil in this designed microcosm experiment.

6.3.5. *Porcellionides pruinosus*

The application of glyphosate, in all three concentrations, did not affect the number of isopods found after the 28 days of exposure; the retrieval rate was very similar to the one observed in control STEM (Fig. 6.6). However, exposure to dimethoate affected the number of isopods retrieved after the exposure period, even at the field dose, less than half of the isopods added in the beginning of the tests were found. Exposure to 5 and 10 times the field dose of dimethoate and the binary mixtures made with the same two concentrations led to a sharp decrease in the number of animals retrieved in the STEM after the 28 days of exposure. Only one isopod was retrieved in the STEM with 5 times the field dose and no isopod was found in the STEM with 10 times the field dose of dimethoate (Fig. 6.6). In the binary mixtures performed the number of isopods recovered decreased with increasing concentrations of the two pesticides: in the mixture made with the field dose 4 isopods were found, in the binary mixture with 5 times the field dose only 2 isopods were found, and in the binary mixture made with the highest concentration of both pesticides no isopod was found (Fig. 6.6).

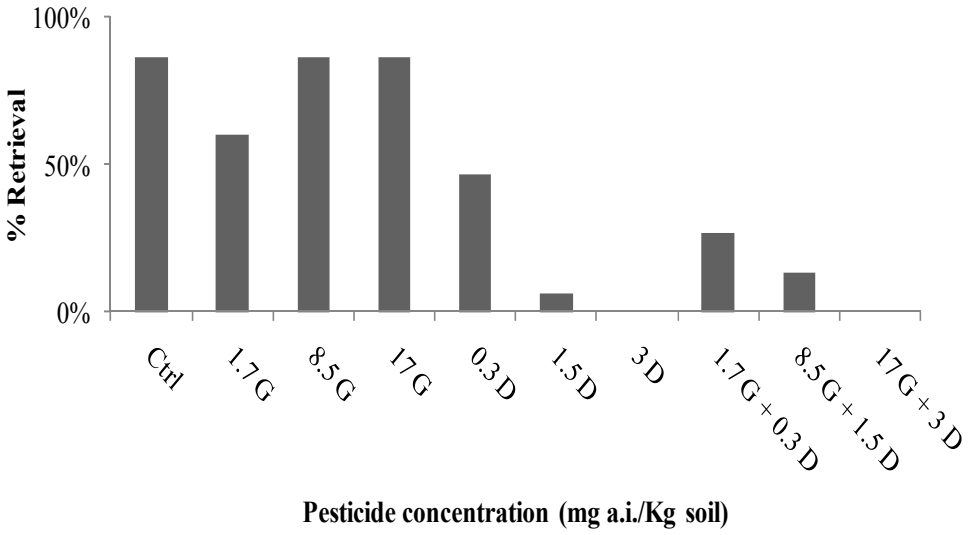

Fig. 6.6 - Percentage of retrieved *Porcellionides pruinosus* in the STEM after 28 d. All units (nominal values) are in mg a.i./Kg soil.

The effects of the insecticide dimethoate on the retrieval rate of the isopod *P. pruinosus* (EC_{50} = 0.46 (0.16-0.80) mg/Kg) was much lower than the concentration responsible for reducing 50 % the survival rate (LC_{50} higher than 75 mg/Kg) of the isopod species *Porcellio scaber* (Fischer et al., 1997), and the species *Porcellio dilatatus* (LC_{50} higher than 20 mg/Kg) exposed for 10 days on a silt-loam soil on a microcosm experiment (Engenheiro et al., 2005). The reason for the discrepancy between the values found in the present study and the works cited above could be the method of pesticide application in the STEM. Since the pesticides were applied using a sprayer after the isopods were in the microcosm, the animals were immediately exposed to the pesticide, which can be considered a more direct and extra route of exposure of dimethoate to the isopods (Sousa et al., 2000; Loureiro et al., 2002).

The assessment of the four biomarkers led to significant differences only on the AChE activity after exposure to dimethoate at field dose and binary mixture and on LPO activity at the field dose of both pesticides and binary mixture (Fig. 6.7). GST activity in the treatments was close to the basal levels observed in control animal, and this detoxifying enzyme does not seem to be triggered by the two pesticides, which has been recorded in previous studies (Jemec et al., 2010). CAT is involved in the reduction of reactive oxygen

species (ROS), and its levels decreased around 66 and 45 % in glyphosate and dimethoate field dose respectively, although no statistical differences were observed. LPO was induced, with statistical differences, at glyphosate and dimethoate field dose concentrations and in the binary mixture. It is known that ROS induces oxidative stress, which could lead to lipid peroxidation (Valant et al., 2009), thus the inhibition in CAT enzymatic levels can be linked with the increase in LPO levels.

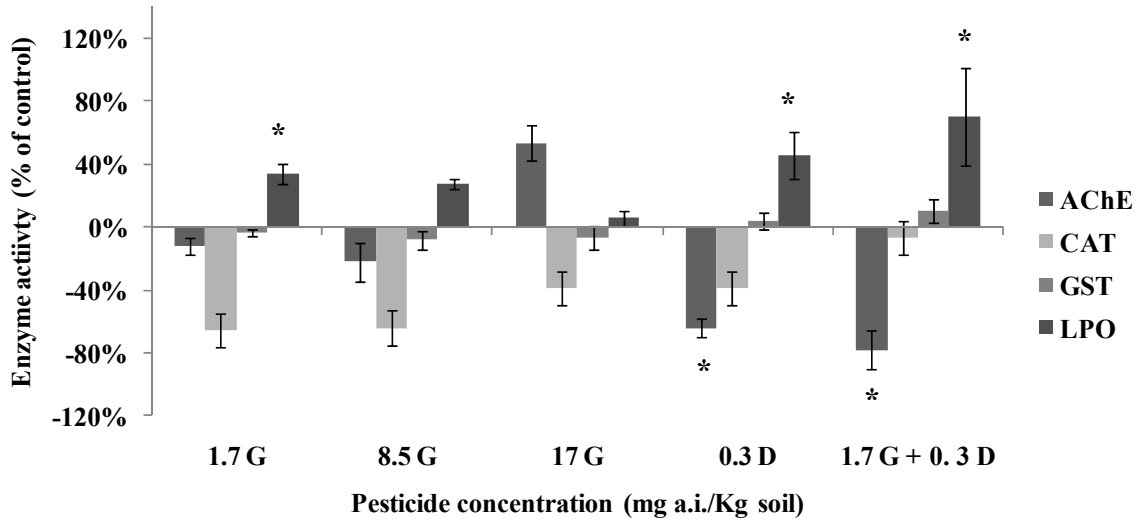

Fig. 6.7 – Variation (as percentage of control) of acetylcholinesterase (AChE), catalase (CAT), glutathione-*S*-transferase (GST), and lipid peroxidation (LPO) activity in *Porcellionides pruinosus* after exposure in the STEM for 28 d. Results are expressed as mean values with standard error. All units of pesticide concentration (nominal values) are in mg a.i./Kg soil. (* indicates statistical differences between control and treatments (Dunnett´s method, $p<0.05$)).

Acetylcholinesterase is responsible for breaking down acetylcholine, and is inhibited by organophosphorous pesticides, since these pesticides bind to the hydroxyl group of the functional part of this enzyme, resulting in an enzyme that has no activity, and ceases to be able to hydrolyze the substrate acetylcholine (Ferreira et al., 2010). AChE inhibition due to exposure to the insecticide dimethoate has been observed in previous studies using terrestrial isopods at sub-lethal concentrations (Engenheiro et al., 2005). This

enzyme sensitivity to sub-lethal concentrations of organophosphorous pesticides could indicate impairment of the regular function of these soil organisms (Loureiro et al., 2005). The inhibition observed in the AChE activity when dimethoate was present in the soil could be associated with the subsequent mortality rate observed in the isopods; since it has been appointed that a reduction in cholinesterase activity up to 50% indicated impairment in the survival capacity of the organisms (Ludke et al., 1974).

6.4 Conclusions

At the field dose, and thus the ecological relevant concentration, no effects were observed in all the endpoints measures in both *B. rapa* (vegetative growth and germination success) and *E. andrei* (vertical distribution and weight variation). The binary mixture approach showed a synergistic effect on shoot length and fresh weight of *Brassica rapa* at 5 times the field dose and antagonism at 10 times the field dose. The effect of the mixtures on the germination success were antagonistic at the field dose (fewer plants germinated than expected), but synergistic at 5 and 10 times the field dose. The decrease in earthworms weight as well as vertical distribution was not statistical different from the control in both single and binary mixtures performed, and in the binary mixtures fewer animals than expected (antagonism) were counted in less contaminated portion of soil. Nevertheless, the decrease in earthworm's weight was correlated with the decrease in bait consumption along the exposure period. There was a high mortality rate observed in the isopods after exposure to dimethoate in the STEM conditions whether in single and binary exposures, clearly demonstrating that an increase in dimethoate concentration led to an increase in the mortality rate. Biomarker changes were observed in AChE when isopods were exposed to dimethoate at field dose and binary mixtures and in LPO when exposed to field doses of the pesticides in single and binary exposure.

References

Bird, R.P., Draper, H.H., 1984. Comparative studies on different methods of malonaldehyde determination. Methods in Enzymology 105, 299 - 305.

Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma 124, 2-22.

Büneman, E.K., Schwenke, G.D., Van –Zwieten, L., 2005. Impact of agricultural inputs on soil organisms – a review. Aust. J. Soil Res. 44, 379-406.

Casablé, N., Piola, L., Fuchs, J., Oneto, M.L., Pamparato, L., Basack, S., Gimenéz, R., Massaro, R., Papa, J.C., Kuten, E., 2007. Ecotoxicological assessment of the effects of glyphosate and chlorpyrifos in an Argentine soya field. J. Soils Sediments 7, 232-239.

Cedergreen, N., Streibig, J.C., 2005. Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? Environmental Toxicology and Chemistry 24, 1676-1683.

Chen, S.K., Edwards, C.A., Subler, S., 2001. A microcosm approach for evaluating the effects of the fungicides benomyl and captan on soil ecological processes and plant growth. Appl. Soil Ecol. 18, 69-82.

Clairborne, A., 1985. Catalase activity. In: Greenwald, R (ed), Handbook of methods for oxygen radical research, CRC Press, Boca Raton, Florida, pp. 283–284.

Dalby, P.R., Baker, G.H., Smith, S.E., 1995. Glyphosate, 2,4, DB and dimethoate: effects on earthworms survival and growth. Soil Biol. Biochem. 27, 1661-1662.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M., Guilhermino, L., 2001. Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. Chemosphere 45, 553-560.

Ellman GL, Courtney KD, Andres VJ, Featherstone R (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmac 7, 88-95

Edwards, C.A., 2002. Assessing the effects of environmental pollutants on soil organisms, communities, processes and ecosystems. Eur. J. Soil Biol. 38, 225-231.

Engenheiro, E.L., Hankard, P.K., Sousa, J.P., Lemos, M.F., Weeks, J.M., Soares, A.M.V.M., 2005. Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environ Toxicol Chem 24, 603–609.

Ferreira, N.G.C., Santos, M.J.G., Domingues, I., Calhôa, C.F., Monteiro, M., Amorim, M.J.B., Soares, A.M.V.M., Loureiro, S., 2010. Basal levels of enzymatic biomarkers and energy reserves in Porcellionides pruinosus. Soil Biol. Biochem. 42, 2128-2136.

Fischer, E., Farkas, S., Hornung, E., Past, T., 1997. Sublethal effects of an organophosphorus insecticide, dimethoate, on the isopod *Porcellio scaber* Latr. Comp. Biochem. Physiol. 116 C, 161-166.

Gange, A.C., Brown, V.K., Farmer, L.M., 1992. Effects of pesticides on the germination of weed seeds: implications for manipulated experiments. J. Appl. Ecol. 29, 3030-310.

Giller, K.E., Beare, M.H., Lavelle, P., Izac, A.-M-. N., Swift, M.J., 1997. Agricultural intensification, soil biodiversity and agroecosystem function. Appl. Soil Ecol. 6, 3-16.

Guilhermino, L, Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M, 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32, 727–738.

Hodge, S., Webster, K.M., Booth, Hepplehwaite, V., O´Halloran, K., 2000. Non-avoidance of organophosohate insecticides by the earthworm *Aporrectodea caliginosa* (Lumbricidae). Soil Biol. Biochem. 32, 425-428.

Huangfu, C.H., Song, X.L., Qiang, S., Zhang, H.J., 2007. Response of wild *Brassica juncea* populations to glyphosate. Pest Manag. Sci. 63, 1133-1140.

ISO (International Organization for Standardization), 2004. Soil quality – Biological methods – Chronic toxicity in higher plants. ISO/DIS 22030, Geneve, Switzerland.

Jemec, A., Drobne, D., Tisler, T. Sepcić, K., 2010. Biochemical biomarkers in environmental studies – lessons learnt from enzyme catalase, glutathione *S*-transferase and cholinesterase in two crustacean species. Environ. Sci. Poll. Res. Int. 17, 571-581.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose–response analysis. Environ. Toxicol. Chem. 24, 2701–2713.

Köneman, W.H., Pieters, M.N., 1996. Confusion of concepts in mixture toxicology. Food Chem. Toxicol. 34, 1025-1031.

Kratz, W., 1998. The bait-lamina test. General aspects, applications and perspectives. Environ. Sci. Pollut. Res. 5, 94-96.

Kools, S.A.E., Boivin, M.-E., Y., Van der Wuff, A.W.G., Berg, M.P., Van Gestel, C.A.M., Van Straalen, N.M., 2009. Assessment of structure and function in metal polluted grasslands using Terrestrial Model Ecosystem. Ecotox. Environ. Safe. 72, 51-59.

Langdon, C.J., Hoson, M.E., Arnold, R.E., Black, S., 2005. Survival, Pb-uptake and behaviour of three species of earthworms in Pb treated soils determined using and OECE-style toxicity test and a soil avoidance test. Environ. Pollut. 138, 368-375.

Lemos, M.F.L., van Gestel, C.A.M., Soares, A.M.V.M., 2010. Developmental toxicity of endocrine disrupters bisphenol A and vinclozolin in a terrestrial isopod. Arch. Environ. Contam. Toxicol. DOI 10.1007/s00244-010-9474-9.

Løkke, H.,Van Gestel, C.A.M., 1998. Handbook of soil invertebrate toxicity tests. Wiley, Chichester.

Loureiro, S., Sousa, J.P., Soares, A.M.V.M., Nogueira, A.J.A., 2005. Assimilation efficiency and toxicokinetics of ^{14}C-lindane in the terrestrial isopod Porcellionides pruinosus: the role of isopods in degradation of persistent soil pollutants. Ecotoxicology 11, 481-490.

Loureiro, S., Soares, A.M.V.M., Nogueira, A.J.A., 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ. Pollut.138, 121-131.

Loureiro, S., Amorim, M., Campos, B., Rodrigues, S., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchystraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157, 625-623

Loureiro, S., Svendsen, C., Ferreria, A.L.G., Pinheiro, C., Ribeiro, F., Soares, A.M.V.M., 2010. Toxicity of three binary mixtures to *Daphnia magna*: comparing chemical modes of action and deviations from conceptual models. Environ. Toxicol. Chem. 29, 1716-1726.

Ludke, J.L., Hill, E.F., Dieter, M.P., 1974. Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. Arch. Environ. Cont. Tox. 3, 1-21.

Lukkari, T., Teno, S., Väisänen, A., Haimi, J., 2006. Effects of earthworms on decomposition and metal availability in contaminated soil: microcosm studies of populations with different exposure histories. Soil Biology and Biochemistry 38, 359-370.

Mäder, P., Fließbach, A., Dubois, D., Gunst, L., Fried, P., Niggli, U., 2002. Soil fertility and biodiversity in organic farming. Science 296, 1694-1697.

Marrs, R.H., William, C.T., Frost, A.J., Plant, R.A., 1989. Assessment of the effects of herbicide spray drift on a range of plant species of conservation interest. Environ. Poll. 59, 71-86.

Matsumura, F., 1987. Comparative metabolism of mixtures of chemicals by animals, plants and microorganisms and their significance in alteration of pollutants tin the environment. In: Methods for assessing the effects of mixtures of chemicals (Eds. Vouk., V.B., Buttler, G.C., Upton, A.C., Parke, D.V., Asher, A.C.) pp. 509-522.

Munshi, S.K., Kumari, A., Sanadi, S., Bakhetia, D.R.C., Sukhija, P.S., 1987. Influence of systemic insecticides on lipid biosynthesis in seeds of Indian mustard (*Brassica juncea* L.). Plant Sci. 53, 201-208.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal e tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351-358.

Olszyk, D., Pfleeger, T., Lee, H.E., Plocher, M., 2010. Phytotoxicity assay for seed production using *Brassica rapa* L. Integrated Environmental Assessment and Management 6, 725-734.

Pestemer, W., Zwerger, P., 1999. Application of a standardized bioassay to estimate the phytotoxic effects of frequently used herbicides on non-target plants. Proceedings XI Symposium Pesticide Chemistry – Human and Environmental Exposure to Xenobiotics. Cremona, Italy, pp. 763–770

Reinecke, A.J., Helling, B., Louw, K., Foursie, J., Reinecke, S.A., 2002. The impact of different herbicides and cover crops on soil biological activity in vineyards in the Western Cape, South Africa. Pedobiologia 46 (5), 475-484.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2010a. Joint effects of three plant protection products to the isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. Chemosphere 80 (9), 1021-1030

Santos, M.J.G., Ferreira, N.G.C., Soares, A.M.V.M., Loureiro, S., 2010b. Toxic effects of molluscicide baits to the terrestrial isopod *Porcellionides pruinosus*. J. Soils Sed. 10 (7), 1335-1343.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., (submitted). Joint effects of three plant protection products to the higher plants *Triticum aestivum* (L.) and *Brassica rapa* (L.)

Sarwar, M., Ahmad, N., Siddiqui, Q.H., Rajput, A.A., 2003. Efficiency of different chemicals on canola strain rainbow (*Brassica napus* L.) for aphids control. Asian J. Plant Sci. 2, 831-833.

Snoo, G.R., Wit, P.J., 1998. Buffer zones for reducing pesticide drift to ditches and risk to aquatic organisms. Ecotox. Environ. Safe. 41, 112-118.

Sousa, J.P., Loureiro, S., Pieper, S., Frost, M., Kratz, W., Nogueira, A.J.A., Soares, A.M.V.M., 2000. Soil and plant diet exposure routes and toxicokinetics of lindane in a terrestrial isopod. Environ. Toxicol. Chem. 19, 2557-2563.

Svendsen, C., Siang, P., Lister, L.J., Rice, A., Spurgeon, S.J., 2010. Similarity, independence, or interaction for binary mixture effects of nerve toxicants for the nematode *Caenorhabditis elegans*. Env. Toxicol. Chem. 29, 1182-1191

Syers, J.K., Springett, J.A., 1984. Earthworms and soil fertility. Plant and Soil 76, 93-104

Van Gestel, C.A.M., Kruidemier, M., Berg, M., 2003. Suitability of wheat straw decomposition, cotton strip degradation and bait-lamina feeding tests determine soil invertebrate activity. Biol. Fertility Soils 37, 115-123.

Valanta, J., Drobne, D., Sepci, Jemec, A., Ksenija Kogej, Kostanjśek, R., 2009. Hazardous potential of manufactured nanoparticles identified by *in vivo* assay. J. Hazard. Mat. 171, 160–165.

Verrel, P., Van Buskir, E., 2004. As the worm turns: *Eisenia fetida* avoids soil contaminated by a glyphosate-bsed herbicide. Bull. Environ. Contam. Toxicol. 72, 219-224

Wauchope, R.D., Buttler, T.M., Hornsby, A.G., Augustin-Beckers, P.W.M., Burt, J.P., 1992. Pesticide properties database for environmental decisionmaking. Rev. Environ. Contam. Toxicol. 123, 1-157.

Zhang, Z.-Y., Zhang, C.-Z., Liu, X.-J., Hong, X.-Y., 2006. Dynamics of pesticide residues in the autumn Chinese cabbage (*Brassica chinensis* L.) grown in open fields. Pest Manage. Sci. 62, 3550-355.

7. General Discussion and Final Considerations

The main aim of this work was to assess the effects of the application of combined plant protection products (PPPs) to non-target organisms. Since in most agricultural fields several PPPs are applied to prevent pest and/or increase crop yield, further investigation of ecotoxicology concerning the impact of PPPs in land and non-target organisms should be conducted in the context of mixture effects.

Biochemical indicators (biomarkers of exposure AChE, GST, CAT and LPO) were assessed in the isopod *Porcellionides pruinosus* after a short-term exposure to metaldehyde and methiocarb, using soil LUFA 2.2 and in an exposure to dimethoate and glyphosate, using a Mediterranean soil in a small-scale terrestrial ecosystem (STEM). In the two evaluations, the enzyme AChE was inhibited after the animals were exposed to the carbamate methiocarb and the organophosphate dimethoate. Even very short exposure periods to methiocarb and longer exposures to the recommended dose of dimethoate led to a significant reduction of this enzyme activity. This seems to confirm the sensitivity of AChE as a warning sign of brain malfunction of the animals correlated with a significant reduction in the survival rate of the isopods. The two enzymes, GST and CAT, involved in protecting cellular damages from oxidative stress, confirmed also to be useful in assessing the effects metaldehyde exposure, whereas LPO levels were induced after exposure to glyphosate and dimethoate in single and binary exposures to the recommended dose. These three biomarkers were useful to evaluate the mechanism of toxicity as well as serve as indicators of the stress condition that these compounds exert on *P. pruinosus.*

The impact on the survival rate of the isopods was significant after a very short exposure period to methiocarb (8 hours) and metaldehyde (50 hours). In the exposure to compounds usually applied as baits, like molluscicides, the use of pre-defined concentrations is not useful or relevant and could be discussed in terms of utility in Environmental Risk Assessment (ERA) procedures. Therefore, and instead of the LC_{50} values usually advised in ERA approaches, the LT_{50} values (as time to achieve lethality of 50% of the organisms) showed to be the best accomplishment to evaluate the effects of baits in real scenario exposures. The impact the molluscicides, applied commonly as baits on the soil surface of agricultural fields, had on the survival of *P. pruinosus* was a clear indication of the toxic effects of these molluscicides, which was undisclosed previously in

terms of effects to terrestrial isopods. The exposure in STEM conditions to 5 and 10 times the field dose of dimethoate during 28 led also to high lethality rates.

The joint toxicity of three binary mixtures performed with dimethoate, glyphosate and spirodiclofen were assessed on the avoidance behaviour of *P. pruinosus* and the reproduction output of *Folsomia candida*, using the two reference models of concentration addition (CA) and independent action (IA). Since the specific modes of action of these three pesticides to the two test-species were unknown, CA and IA were used as equally valid reference model to evaluate mixture toxic effects.

The isopods escaped from the contaminated section of the test-boxes with increasing concentrations of all PPPs, showing avoidance behaviour towards concentrations higher than the recommended application dose (AC_{50} was always higher than the predicted environmental concentration). Synergism was observed only in just one combination (glyphosate with spirodiclofen applied to *P. pruinosus*) after fitting the IA model to data. In mixture ecotoxicology, synergism is considered the worrying condition because it means enhancement of toxicity (in this case, enhanced escape response).

In an overall look of the results of the toxic effects of the binary mixtures, the similitude of predictions between CA and IA was very consistent, namely on the evaluation of the effects of mixtures on collembolan reproduction. Thus, the information gathered from fitting both models to data render predictions that were complementary and never contradictory.

Comparing the toxicity results from binomial data of isopods avoidance behaviour and continuous data of collembolan the reproduction success, led to a difference in the threshold values, hence the AC_{50} values of glyphosate and dimethoate the median values were more than 100-times higher than the EC_{50} calculated for the collembolan reproduction. One reason that might explain such a difference would be the methodology used, since isopods can escape to an uncontaminated soil in the test-boxes, whereas collembola are enclosed in the contaminated medium through all the exposure period. Furthermore, the specific sensitivity of each species to the referred toxicants should be stresses out as a factor responsible for the very different effect levels observed in the two organisms.

The joint toxicity of three binary mixtures performed with dimethoate, glyphosate and spirodiclofen was also assessed on the fresh weight and shoot length of *Triticum aestivum* and *Brassica rapa*. In both species, plants grew uniformly along with increasing concentrations of PPP in the soil until the highest concentration was applied to the soil, where significant effects were observed in the two endpoints. Thus, only at high concentrations of the three pesticides the growing ability of the two plant species was impaired. This could be one reason for the similar EC_{50} calculated for the single exposures to the three PPP in these two plants (differences were never above a factor of two).

In general terms, fitting data in CA and IA models, led to similar conclusions about the toxic effects of the mixtures. The only exception was when data from glyphosate and dimethoate applied to *T. aestivum*, derived antagonism at low doses following CA model, and synergism when glyphosate was dominant in the mixture after IA model. Subsequent fitting of the data according to the two deviations allowed arguing that synergism would be the deviation that explained better the data. This apparent contradiction may be related to the specific mode of action of the pesticides in some of the binary mixtures, where a small change in the quantity of one of the pesticides can lead to changes in plant growth. Nevertheless, there was a general concordance in the outcome of fitting the models to data, evidencing that when submitted to the same binary mixture comparable conclusions could be derived from these two plant species.

A small-scale terrestrial ecosystem (STEM) was used to evaluate the combined effect of spirodiclofen and dimethoate in the growth of *B. rapa* and the weigth variation and vertical distribution of *E. andrei*. Regarding the effects on *B. rapa*, they were not enough to derive an effect level, although a concomitant decrease in length and weight was observed with increasing pesticide concentrations. Behavioural results of joint PPP application gave more information on possible detrimental effects on the earthworms, and further implication to the soil ecosystem; hence the absence of worms could indicate a loss to the normal regulation of ecosystem in terms of nutrient recycling.

In the second STEM experiment made with a binary mixture of glyphosate and dimethoate, in addition to the cited species, *P. pruinosus* and bait-lamina strips were added to each microcosm. The endpoints evaluated in *B. rapa* decreased with increasing concentrations of glyphosate in soil, and statistical differences observed at 5 and 10 times

the field dose on fresh weight and germination success. In the binary mixtures made 5 and 10 times the field dose of both pesticides a statistically significant decrease was observed in plants fresh weight and length. Thus, change between the two deviations (synergism and antagonism) seems to indicate that if the dose of the pesticides in the mixture is altered, this may lead to different effects in the growth pattern of the plants. No differences were observed on worms' vertical distribution or weight change in any of the treatments made, although the number of baits eaten decreased significantly at the highest dose of dimethoate and on the three binary mixtures performed. A correlation between the decreasing number of eaten baits and the increasing amount of weight loss in earthworms could be established. Dimethoate was the pesticide responsible for the synergism in all the binary mixtures regarding bait-consumption (fewer baits eaten than expected), which can represent impairment in the function of the soil.

In an overall perspective, STEM was a good tool to assess the environmental problems derived from the application of combined pesticides to non-target organisms. Microcosms are surrogates of real scenarios of exposure in agricultural fields, and the inclusion of several species, provide more information about the effects of toxic mixtures at several ecological levels. The effects observed ranged from cellular damages (biomarkers of exposure) to growth impairment in organisms (earthworms and plants) and functional stability of the soil (bait-lamina test).

Discussing the results of the joint toxicity of mixtures should be made alongside with the knowledge of the precise mode of toxic action of the pesticides inside the organisms, in order to make more assertive assumptions about the mechanisms involved in possible interactions between PPPs. Due to the general lack of the systematic mechanisms underlying the specific toxic effects of PPPs, only some considerations could be made about the effects of the mixtures tested to the organisms.

An encompassing perspective should be made regarding the several test-trials made with the three PPPs on the non-target organisms. The two reference models explained the toxicity of one-quarter of the test trials, and in some occasions CA and IA proved to be successfully explaining the effects of theoretical both similar and dissimilar acting compounds. Only in four binary mixtures synergism occurred, however the synergistic predictions were never observed after fitting the two models to the same data (i.e.

synergism following fitting CA to data was not corroborated by fitting IA or vice-versa). Antagonism was the interaction described in the majority of the test-trials; in almost two-thirds of the test-trials the effects observed were bellow the effect predicted by the responses observed in the single exposures. In the STEM tests, again antagonism was the main interaction observed (more than two-thirds of the interaction observed) whereas synergism was observed in less than one-third of the test-trials. Synergism seemed to be evidently showed in the bait-lamina consumption since the effects observed were much higher than expected from the single exposures.

The predominance of antagonism under different methodologies and test-species seemed to confirm that the absence of enhanced toxicity (albeit not the absence of effects) was the general rule in the toxic effects of the three PPP used. The search for synergism is one of the main objectives in ecotoxicological assessment of joint effects, given that the increasing toxicity of mixtures of chemicals, sometimes below their threshold toxic levels, could pose a threat to exposed organisms. However, the systematic prevalence of antagonism in most published studies, including the ones present in this thesis, appears to confirm that synergism when testing PPPs application to edaphic organism seldom is observed. As it was stated previously a strong synergistic effect was observed on bait-lamina consumption, hence the effects observed were much stronger than expected. Since this method reflect soil function, it seems pertinent to highlight the consistent synergism rendered by this methodology.